Endocrinology. First published ahead of print October 30, 2009 as doi:10.1210/en.2009-1036

Androgen Regulates Brain-Derived Neurotrophic Factor in Spinal Motoneurons and Their Target Musculature

Tom Verhovshek, Yi Cai, Mark C. Osborne, and Dale R. Sengelaub

Department of Psychological and Brain Sciences and Program in Neuroscience, Indiana University, Bloomington, Indiana 47405

Trophic factors maintain motoneuron morphology and function in adulthood. Brain-derived neurotrophic factor (BDNF) interacts with testosterone to maintain dendritic morphology of spinal motoneurons. In addition, testosterone regulates BDNF's receptor (trkB) in motoneurons innervating the quadriceps muscles as well as in motoneurons of the highly androgen-sensitive spinal nucleus of the bulbocavernosus (SNB). Given these interactive effects, we examined whether androgen might also regulate BDNF in quadriceps and SNB motoneurons and their corresponding target musculature. In both motoneuron populations, castration of males reduced BDNF immunolabeling, and this effect was prevented with testosterone replacement. ELISA for BDNF in the target musculature of quadriceps (vastus lateralis, VL) and SNB (bulbocavernosus, BC) motoneurons revealed that BDNF in the VL and BC muscles was also regulated by androgen. However, although castration significantly decreased BDNF concentration in the VL muscle, BDNF concentration in the BC muscle was significantly increased in castrates. Treatment of castrated males with testosterone maintained BDNF levels at those of intact males in both sets of muscles. Together, these results demonstrate that androgens regulate BDNF in both a sexually dimorphic, highly androgen-sensitive neuromuscular system as well as a more typical somatic neuromuscular system. Furthermore, in addition to the regulation of trkB, these studies provide another possible mechanism for the interactive effects of testosterone and BDNF on motoneuron morphology. More importantly, by examining both the motoneurons and the muscles they innervate, these results demonstrate that within a neural system, BDNF levels in different components are differentially affected by androgen manipulation. **(*Endocrinology* 151: 0000–0000, 2010)**

Gonadal steroids exhibit a wide array of neuroprotective and neurotherapeutic effects (1–3). Androgens have powerful neuroprotective effects; for example, testosterone protects against cell death in cultured hippocampal neurons (4) and injury-induced dendritic atrophy in cortical pyramidal cells (5). Androgens also exhibit a wide array of neuroprotective and neurotherapeutic effects in motoneurons, including supporting cell survival, axonal regeneration, and dendritic maintenance (6).

Previous work has shown combinatorial treatment effects of testosterone and brain-derived neurotrophic factor (BDNF). BDNF is a member of the neurotrophin family and promotes motoneuron survival and axon outgrowth (7). In motoneurons of the spinal nucleus of the bulbocavernosus (SNB), a sexually dimorphic and highly androgen-sensitive motor population in the lumbar spinal cord of rats (8), treatment with testosterone and BDNF has an interactive effect on the regulation of androgen receptor expression (9) as well as dendritic length (10). Combined treatment with both testosterone and BDNF is more effective than treatment with either compound alone in the maintenance of androgen receptor immunoreactivity in axotomized SNB motoneurons (9). Similarly, although treatment with either compound alone is ineffective, dendritic lengths in axotomized SNB motoneurons are supported by combined treatment with both testosterone and BDNF (10).

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2009-1036 Received September 1, 2009. Accepted October 8, 2009.

Abbreviations: BC, Bulbocavernosus; BDNF, brain-derived neurotrophic factor; BHRP, HRP conjugated to the cholera toxin B subunit; HRP, horseradish peroxidase; LSD, least significant difference; NS, not significant; SNB, spinal nucleus of the bulbocavernosus; VL, vastus lateralis; wwt, wet weight of tissue.

Testosterone and BDNF could interact in a variety of ways. BDNF and its high-affinity receptor, trkB, are present in SNB motoneurons (11, 12). Expression of mRNA for BDNF and trkB in SNB motoneurons is regulated by the presence of androgens (12). In addition, we have demonstrated that testosterone regulates trkB receptors in SNB motoneurons (11). BDNF and its receptor are also present in a variety of other spinal motoneurons (13), including those innervating the quadriceps muscle (14), and we have also demonstrated that testosterone regulates trkB in quadriceps motoneurons (11). Because gonadal steroids regulate BDNF in several brain structures (15–17), we hypothesized that testosterone could have a similar regulatory effect on BDNF in SNB and quadriceps motoneurons.

BDNF is also present in skeletal muscle (18, 19), including the target musculature of the SNB (20) and quadriceps (14) motoneuron populations. Hormonal regulation of BDNF protein expression has been demonstrated in accessory male genital tissues (21); therefore, we further hypothesized that testosterone could have a similar regulatory effect on BDNF in the bulbocavernosus (BC) and quadriceps muscles. Importantly, unlike previous studies in individual neural or somatic structures, by examining both motoneurons and the muscles they innervate, we can assess the potential androgenic regulation of BDNF across different components of a defined neural system.

Materials and Methods

Adult male Sprague Dawley rats (approximately 100 d old; Harlan, Indianapolis, IN) were maintained on a 12-h light, 12-h dark cycle, with unlimited access to food and water. Animals were castrated under isoflurane anesthesia and immediately given sc, interscapular implants of SILASTIC Brand (Dow Corning, Midland, MI) tubing filled with testosterone (4-androsten-17β-ol-3-one; Steraloids, Newport, RI; 3.18 mm outer diameter, 1.57 mm inner diameter, 45 mm long) or left blank. These testosterone implants produce plasma titers of testosterone in the normal physiological range (22). A group of age-matched, untreated, gonadally intact normal males were also included. Each of the resulting three groups was further subdivided for examination of either SNB (normal, n = 6; castrate, n = 6; castrate + testosterone, n = 6) and quadriceps motoneurons (normal, n = 5; castrate, n = 5; castrate + testosterone, n = 5), or BC muscle (normal, n = 6; castrate, n = 6; castrate + testosterone, n = 6) and vastus lateralis (VL) muscle of the quadriceps (normal, n = 8; castrate, n = 8; castrate + testosterone, n = 8). All procedures were carried out in accordance with the Indiana University Animal Care and Use Guidelines.

Motoneuron immunohistochemistry

Motoneuron identification

In rats, SNB motoneurons can be unambiguously identified as large, darkly staining, multipolar cells confined to a discrete, medially located nucleus in the L5–S1 segments of the spinal cord. In contrast, the motoneurons innervating the quadriceps muscles do not form a discrete nucleus but instead are contained within a large continuous population of motoneurons in the lateral motor column, specifically in column 3 in the L2 spinal segment of the lateral motor column (14, 23). Thus, to identify quadriceps motoneurons for subsequent immunohistochemical analysis, the appropriate location in the lateral motor column was first identified by retrograde labeling using horseradish peroxidase (HRP) conjugated to the cholera toxin B subunit (BHRP; List Biological Laboratories, Campbell, CA) as previously described in Osborne *et al.* (11). Two days before they were killed, animals were anesthetized with isoflurane, and the left VL muscles were exposed and injected with 2 μl of a 0.2% solution of BHRP.

Four weeks after hormonal manipulations, a period sufficient to observe hormone-mediated morphological changes in motoneurons (24), animals were weighed, overdosed with urethane (0.25 g/100 g body weight), and perfused intracardially with 0.1 M sodium PBS (pH 7.2), followed by cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2). The L2 or L5–S1 segments were postfixed individually in the same fixative for 18 h and then transferred to 30% sucrose in 0.1 M sodium phosphate buffer (pH 7.4) overnight for cryoprotection. The following day, spinal cords were sectioned horizontally into three alternate series at 30 μm and stored in 0.1 M sodium phosphate buffer. To identify sections containing SNB motoneurons, one series of sections was immediately mounted on gelatin-coated slides and stained with neutral red. These sections were then examined under light microscopy for the presence of SNB motoneurons, and the appropriate matching sections in the alternate series were selected for immunohistochemical processing. To identify the portion of the lateral motor column containing quadriceps motoneurons, BHRP-labeled quadriceps motoneurons were visualized immediately in one series of sections using a modified tetramethyl benzidine protocol (25), mounted on gelatin-coated slides, and counterstained with neutral red (see Ref. 11 for details). The location of BHRP-labeled motoneurons in each animal was then plotted using a computer-based morphometry system (Stereo Investigator; MBF Bioscience, Williston, VT). The appropriate matching sections in the alternate series were selected for immunohistochemical processing.

Immunohistochemistry

To control for potential processing differences, sections from animals from each group were processed simultaneously in the same solutions through all steps. Sections were rinsed with 0.01 M PBS and incubated 30 min in 50% methanol. Sections were then incubated 30 min in 0.5% hydrogen peroxide in a blocking solution containing 1% normal goat serum and 0.1% Triton X-100 in 0.01 M PBS, followed by 1 h in blocking solution without hydrogen peroxide. Sections were then incubated 48 h at 4 C in rabbit anti-BDNF primary antibody (1:500 dilution, SC-546; Santa Cruz Biotechnology, Santa Cruz, CA) followed by 24 h incubation in biotinylated goat antirabbit secondary antibody (1:200; Vector Laboratories, Burlingame, CA). Antibody signal amplification was achieved via an ABC reaction (Vector Standard Elite Kit) and visualized with 0.035% diaminobenzidine and 0.006% H_2O_2. Sections were then mounted onto gelatin-coated slides, defatted, and coverslipped. Control sections incubated without primary antibody were generated and showed virtually no immunostaining.

We used a previously described semiquantitative method to assign cells to categories of intense, moderate, or light immunostaining (11). Briefly, motoneurons were identified morphologically as cells with large multipolar somata. After outlining the perimeter of individual motoneuron somata, the OD of immunolabeling in each soma was measured (Stereo Investigator) as average luminosity per pixel (in 256 gray levels, where black = 0 and white = 256; a mean of approximately 7300 and 6400 pixels per soma, for quadriceps and SNB motoneurons, respectively, were assessed) at a final magnification of ×1480 under bright-field illumination. To control for spurious differences in immunostaining across sections and animals, OD measures within each section were expressed relative to immunostaining in the adjacent white matter. Although spinal cord white matter contains BDNF (26), BDNF expression is greater in spinal cord gray matter compared with white matter (27). More importantly, pilot studies showed that the intensity of BDNF immunolabeling in the white matter did not vary with hormone condition and thus provided a reliable reference measure of local immunostaining. Immunostaining of motoneuron somata was then categorized as being either intense (having a relative OD of at least 1 SD below the mean of normal males), moderate, or light (having a relative OD of at least 1 SD above the mean of normal males). Importantly, this method does not attempt to assess the actual amount of BDNF in motoneurons but rather simply provides a reliable method for categorizing cells by immunostaining intensity for subsequent frequency analyses.

For the SNB, motoneurons were identified by their characteristic location in the L5–S1 spinal segment, and all medially located, large, multipolar somata were assessed on both the left and right sides, regardless of the density of immunostaining. For quadriceps motoneurons, within each animal, the rostrocaudal range through the lateral motor column that contained the relevant motoneurons was determined using the location of BHRP-labeled motoneurons as described above. For each animal, the rostrocaudal limits were then superimposed on the matching immunostained sections using Stereo Investigator, and the OD of immunolabeling of all motoneurons in the relevant portion of the lateral motor column of the immunohistochemically processed sections was then assessed. Only cells with large multipolar somata in the lateral motor column were assessed, again regardless of the density of immunostaining. To prevent confounding of immunolabeling with BHRP labeling, immunostained motoneuron somata were sampled contralateral to the BHRP injection. Because the BHRP labeling of quadriceps motoneuron somata and dendrites is completely unilateral, such labeling does not compromise contralateral immunolabeling. The cross-sectional area of each motoneuron soma sampled was also recorded for both the SNB and the quadriceps populations.

Muscle BDNF assay

Muscle extraction and sample preparation

Four weeks after hormonal manipulations, animals were killed via rapid decapitation, and BC and left VL muscles were removed. Gross muscle weights were measured to confirm hormonal manipulations, and the muscles were then cut into smaller samples (~100 mg), snap frozen on dry ice, and then stored at −70 C. Samples were homogenized in an ice-cold RIA precipitation (RIPA) buffer (Sigma-Aldrich, St. Louis, MO; 1:10 wt/vol) containing a peroxidase inhibitor cocktail (5 μl/ml RIPA buffer; Sigma) and then centrifuged at 14,000 × *g* at 4 C for 30 min. The resulting supernatants were decanted and stored overnight at −70 C.

ELISA

The amount of BDNF in BC and VL muscles was quantified using a Quantikine BDNF ImmunoAssay Kit (R&D Systems, Minneapolis, MN). The ELISA was performed according to the manufacturer's recommendations. Briefly, 96-well microplates, precoated with mouse monoclonal BDNF antibody, were incubated 2 h with BDNF standards, control solutions, and muscle tissue sample supernatants. Microplates were then incubated 1 h with mouse monoclonal antibody conjugated to HRP. After this incubation, the microplates were thoroughly washed three times with wash buffer and then incubated 30 min in tetramethyl benzadine substrate solution. The reaction was stopped using a sulfuric acid stop solution, and the absorbance of each well was measured immediately at 450 nm (with a wavelength correction set at 570 nm) using a Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA). BDNF concentrations were then calculated by SOFTmax PRO software (Molecular Devices) and expressed as picograms BDNF per 100 mg wet weight of tissue (wwt). The mean intraassay coefficient of variation was 6.43%. To control for intraassay variability, BDNF standards were assayed in duplicate and muscle sample supernatants assayed in quadruplicate. The interassay coefficient of variation was 7.87%. Data are presented as mean (±SEM) BDNF concentration per 100 mg wwt of quadruplicate ELISA determinations for each muscle.

Total protein assay

To rule out a general effect of hormonal manipulation on muscle protein, the amount of total protein in BC and VL muscles was quantified using a DC Protein Assay kit (Bio-Rad, Hercules, CA). Samples were taken from the same supernatants collected for the ELISAs and assayed according to the manufacturer's recommendations. Briefly, standards and samples were pipetted into a clear 96-well microplate. A solution containing alkaline copper tartrate, Folin reagent, and sodium dodecyl sulfate was then added to each well. After 15 min incubation, the absorbance of each well was measured at 750 nm using a Spectramax 190 plate reader. The protein concentration of each standard and sample was calculated using SOFTmax PRO software and expressed as milligrams of total protein per 100 mg wwt. The mean intraassay coefficient of variation was 1.82%. To control for intraassay variability, BDNF standards and muscle sample supernatants were run in duplicate. The interassay coefficient of variation was 6.43%. Data are presented as mean (±SEM) total protein concentration per 100 mg wwt of duplicate assay determinations for each muscle from each experimental animal.

Statistics

ANOVA followed by appropriate planned comparisons [Fisher's protected least significant difference (LSD)] were used in cases of true interval data (*e.g.* muscle weight, cell number or size, and protein concentration). For cases where the data were ordinal in nature (density of immunolabeling), analyses consisted of the nonparametric Kruskal-Wallis ANOVA by ranks. The number of immunolabeled cells within each intensity category was analyzed using a two-way ANOVA with repeated measures (group by label, with label as the repeated factor). For presentation purposes, digital light micrographs were obtained

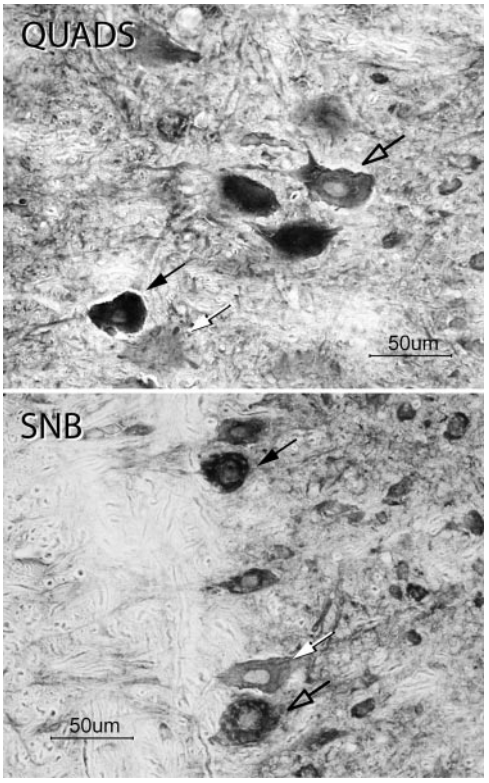

FIG. 1. Digital light micrographs of horizontal sections of regions of the spinal cord containing quadriceps (QUADS; *top*) and SNB (*bottom*) motoneurons from a normal male after immunolabeling for BDNF. Intensely (*black arrows*), moderately (*open arrows*), and lightly (*white arrows*) immunostained somata are indicated. *Scale bars*, 50 μm.

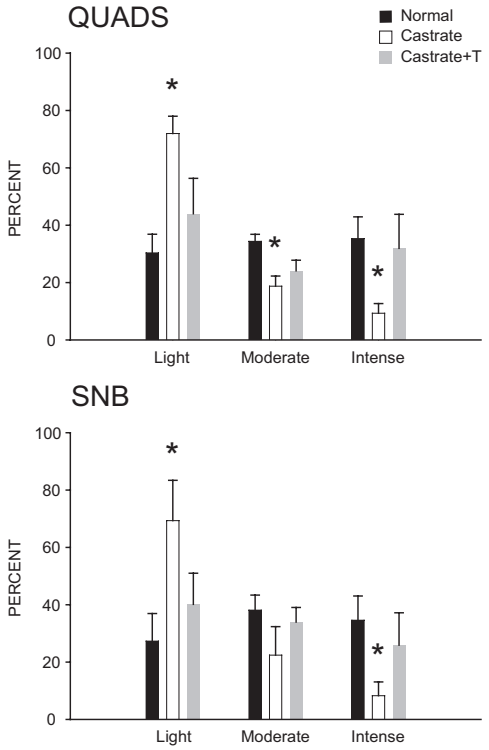

FIG. 2. Histograms of the number of intensely, moderately, and lightly immunostained quadriceps (QUADS; *top*) or SNB (*bottom*) motoneuron somata after immunolabeling for BDNF in normal males (*black bars*), castrated males (*white bars*), and castrated males treated with testosterone (T; *gray bars*). Bar heights represent means ± SEM. *, Different from normal males.

using an MDS 290 digital camera system (Eastman Kodak Co., Rochester, NY). Brightness and contrast of these images were adjusted in Adobe Photoshop.

Results

Quadriceps neuromuscular system

Immunohistochemistry

Mean (±SEM) OD of the white matter samples was 112.5 (±3.50) and did not differ across hormonal condition [Kruskal-Wallis $H_{(2)} = 0.98$, not significant (NS)]. Immunostaining for BDNF resulted in pronounced labeling in quadriceps motoneurons (Fig. 1). The OD of an average of 64.53 ± 3.72 quadriceps motoneuron somata was assessed over an average of 2.87 ± 0.37 sections per animal; the number of motoneurons sampled did not differ across groups [$F_{(2,12)} = 0.31$, NS].

In normal males, the mean of the relative OD of quadriceps motoneuron somata immunolabeled for BDNF was 0.44 with a SD of 0.05. Intensely immunostaining somata comprised 35.3% of the population; moderately immunostaining somata comprised 34.4% of the population; and lightly immunostaining somata comprised the remaining 30.3% (Fig. 2).

BDNF immunolabeling in quadriceps motoneurons was sensitive to hormonal condition [$F_{(4,24)} = 4.53$; $P < 0.05$]. The frequency of intensely immunolabeled quadriceps motoneurons in castrated males was decreased by 66.3% (LSD, $P < 0.05$); intensely immunolabeled quadriceps somata then comprised only 9.3% of the population (Fig. 2). The frequency of moderately immunolabeled quadriceps somata also differed across groups and in castrated males was decreased by 47.4% (LSD, $P < 0.05$); moderately immunolabeled quadriceps somata then comprised only 18.7% of the population (Fig. 2). This decrease in the frequency of intensely and moderately immunolabeled somata was countered by a change in the frequency of lightly immunolabeled somata. In castrated males, the frequency of lightly immunolabeled somata increased by 138% and then comprised 72.0% of the quadriceps population (LSD, $P < 0.05$; Fig. 2).

Changes in the frequencies of immunolabeled quadriceps motoneurons after castration were prevented by testosterone replacement. The frequencies of intensely (31.9%), moderately (24.1%), and lightly (44.0%) immunostaining somata in castrated males treated with testosterone were similar to those of normal males [$F_{(2,16)} = 0.74$, NS; Fig. 2].

The size of quadriceps motoneuron somata did not change significantly with hormonal condition [$F_{(2,12)} =$

1.33, NS]. Sizes of somata of quadriceps motoneurons in untreated castrated males (434.75 ± 14.71 μm^2) were 83% of those in intact males (520.88 ± 44.56 μm^2; LSD, NS), whereas those of testosterone-treated males (512.95 ± 53.96 μm^2; LSD, NS) were 98% of those in intact males.

BDNF ELISA and total protein assay

The weights of VL muscles in normal males were 1.55 ± 0.08 g. As expected, the weight of VL muscle did not change significantly with hormonal condition; VL muscles in untreated castrated males (1.57 ±.08 g) and testosterone-treated castrated males (1.51 ± 0.09 g) did not differ from those of normal males [$F_{(2,21)} = 0.74$, NS].

BDNF concentrations in VL muscles averaged 16.80 ± 2.47 pg/100 mg wwt across all treatment groups, similar to previous findings (18, 19). In normal males, BDNF concentrations were 24.25 ± 4.81 pg/100 mg wwt. BDNF concentrations in the VL muscles were sensitive to hormonal manipulation [$F_{(2,21)} = 4.66$; $P < 0.05$]. BDNF concentrations in castrated males (8.29 ± 2.28 pg/100 mg wwt) decreased 66% compared with those of normal males (LSD, $P < 0.05$) and 54% compared with testosterone-treated castrated males (17.87 ± 3.63 pg/100 mg wwt), but this difference failed to reach significance (LSD, NS). BDNF concentrations in normal males and testosterone-treated castrated males did not differ from each other (LSD, NS; Fig. 3).

Because these changes in BDNF concentrations could reflect a change in overall protein levels consequent to hormonal manipulation, we examined whether castration or testosterone treatment affected total protein concentration in the VL muscles. Total protein concentrations in VL muscles averaged 9.82 ± 0.31 pg/100 mg wwt across all treatment groups, similar to previous findings (28). Total protein concentration in VL muscle of normal males was 10.40 ± 0.63 mg/mg wwt. In contrast to BDNF concentration, total protein concentration in the VL was not sensitive to hormonal manipulation [$F_{(2,21)} = 0.85$, NS]. The mean concentration of total protein in VL muscles in castrated males (9.55 ± 0.47 mg/mg wwt) and testosterone-treated castrated males (9.52 ± 0.52 mg/mg wwt) was similar to that of normal males (LSD, NS).

SNB/BC neuromuscular system

Immunohistochemistry

Mean OD of the white matter samples was 106.79 (±1.54) and did not differ across hormonal condition [Kruskal-Wallis $H_{(2)} = 0.98$, NS]. Immunostaining for BDNF resulted in pronounced labeling in SNB motoneurons (Fig. 1). The OD of an average of 45.56 ± 3.80 SNB motoneuron somata was assessed over an average of 2.56 ± 0.28 sections per animal; the number of motoneurons sampled did not differ across groups [$F_{(2,15)} = 0.99$, NS].

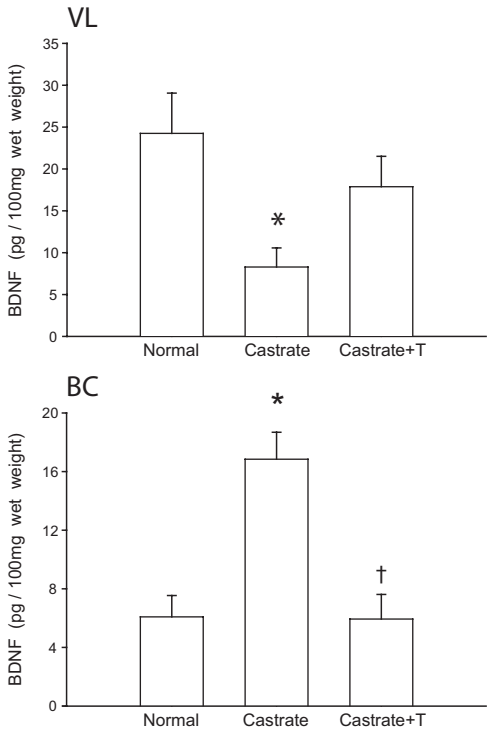

FIG. 3. BDNF protein concentration (picograms per 100 mg wwt) in VL muscle (*top*) and BC muscle (*bottom*) by ELISA determination in normal males, castrated males, and castrated males treated with testosterone (T). Bar heights represent means ± SEM. *, Different from normal males; †, different from castrated males.

In normal males, the mean of the relative OD of SNB motoneuron somata immunolabeled for BDNF was 0.41 with a SD of 0.06. Intensely immunostaining somata comprised 34.6% of the population; moderately immunostaining somata comprised 38.1% of the population; lightly immunostaining somata comprised the remaining 27.3% (Fig. 2).

As in the quadriceps motoneurons, BDNF immunolabeling was sensitive to hormonal condition [$F_{(4,30)} = 2.72$; $P < 0.05$]. The frequency of intensely immunolabeled SNB motoneuron somata in castrated males was decreased by 66.2% (LSD, $P < 0.05$); intensely immunolabeled SNB somata then comprised only 8.3% of the population (Fig. 2). This decrease in the frequency of intensely immunostaining somata was countered by a change in the frequency of lightly immunostaining somata. In castrated males, the frequency of lightly immunostaining somata increased by 135% (LSD, $P < 0.05$) and then comprised 69.3% of the SNB population (Fig. 2). The frequency of moderately immunolabeled SNB somata did not differ across groups (LSD, NS).

As in the quadriceps motoneurons, changes in the frequencies of immunolabeled SNB motoneurons after castration were prevented by testosterone replacement. The

frequencies of intensely (25.9%), moderately (34.0%), and lightly (40.1%) immunostaining somata in castrated males treated with testosterone were similar to those of normal males (LSD, NS; Fig. 2).

The size of SNB motoneuron somata is androgen-sensitive (29), and as expected, the mean cross-sectional area of SNB somata differed across groups [$F_{(2,15)} = 3.77$; $P < 0.05$]. Sizes of somata of SNB motoneurons in untreated castrated males (369.17 ± 19.33 μm^2) were reduced to 77.2% of those in normal males (478.35 ± 95.05 μm^2; LSD, $P < 0.05$). Also as expected, decreases in the size of SNB somata after castration were prevented with testosterone replacement, and testosterone-treated males had soma sizes (442.75 ± 24.29 μm^2) that were 92.6% of those of normal males (LSD, NS).

BDNF ELISA and total protein assay

The weights of the BC muscle in normal males were 0.81 ± 0.02 g. As expected, the weight of the BC muscle was sensitive to hormonal manipulation [$F_{(2,15)} = 306.38$; $P < 0.05$]. The weights of BC muscle in untreated castrated males (0.26 ± 0.01 g) were 32% of those in intact males (LSD, $P < 0.05$), whereas those of testosterone-treated males (0.92 ± 0.03 g) were 114% of those in intact males (LSD, $P < 0.05$).

BDNF concentrations in BC muscles averaged 9.63 ± 1.53 pg/100 mg wwt across all treatment groups, similar to previous findings (18, 19). In normal males, BDNF concentration was 6.09 ± 1.46 pg/100 mg wwt, which were 75% smaller when compared with the BDNF concentration observed in the VL (LSD, $P < 0.05$). As found with the VL muscle, BDNF concentrations in the BC muscles were sensitive to hormonal manipulation [$F_{(2,15)} = 14.10$; $P < 0.05$]. However, unlike the VL muscle, BDNF concentrations in the BC muscle of castrated males (16.85 ± 1.84 pg/100 mg wwt) were increased 177% compared with normal males (LSD, $P < 0.05$) and 184% compared with testosterone-treated castrated males (5.94 ± 1.68 pg/100 mg wwt; LSD, $P < 0.05$). BDNF concentrations in normal males and testosterone-treated castrated males did not differ from each other (LSD, NS; Fig. 3).

Total protein concentrations in BC muscles averaged 8.62 ± 0.42 pg/100 mg wwt across all treatment groups, similar to previous findings (28). In normal males, total protein concentration was 9.51 ± 0.94 mg/mg wwt and did not differ from the total protein concentration observed in the VL (LSD, NS). Similar to VL muscles, total protein concentration in the BC muscle was not sensitive to hormonal manipulation [$F_{(2,15)} = 1.67$, NS]. The mean concentration of total protein in BC muscles in castrated males (7.72 ± 0.46 mg/mg wwt) and testosterone-treated castrated males (8.64 ± 0.59 mg/mg wwt) was similar to that of normal males (LSD, NS).

Discussion

Consistent with previous findings demonstrating steroid hormone regulation of BDNF in several brain structures (15–17), autonomic neurons (30), and peripheral tissues (21), we showed that BDNF is regulated by androgen both centrally and peripherally in two different neuromuscular systems. Importantly, this androgenic regulation was observed in both a sexually dimorphic, highly androgen-sensitive neuromuscular system as well as a more typical somatic neuromuscular system, demonstrating that hormonal effects on BDNF are not limited to traditional gonadal hormone targets. Castration reduced BDNF immunolabeling in both quadriceps and SNB motoneurons. Interestingly, although BDNF was also decreased in the VL muscle of castrated males, BDNF levels were increased in the BC muscle after castration. Total protein concentrations in both the VL and BC muscles were not affected by castration. Thus, the changes we observed in BDNF concentration in the muscles were not the result of a general change in protein levels. Treatment with testosterone maintained BDNF in both quadriceps and SNB neuromuscular systems.

In both the typical somatic motoneurons innervating the quadriceps and the highly androgen-sensitive motoneurons of the SNB, castration decreased the frequency of intensely immunolabeled and increased the frequency of lightly BDNF-immunolabeled somata. Our results are consistent with those of Ottem *et al.* (12) showing that castration decreases BDNF mRNA and protein in SNB motoneurons. Thus, the decrease in the frequency of intensely BDNF-immunolabeled SNB motoneurons may reflect a decrease in BDNF production in the motoneurons consequent to a decrease in BDNF mRNA expression. The presence of BDNF mRNA has also been demonstrated in other motoneuron populations including those innervating the quadriceps muscle (14, 31), and the decrease in BDNF immunolabeling of quadriceps motoneurons may similarly reflect a decrease in BDNF production. However, Ottem *et al.* (12) found no effect of castration on BDNF message or protein in the less androgen-sensitive motoneurons of the retrodorsolateral nucleus, a population of somatic motoneurons innervating the intrinsic muscles of the foot. Although this could simply reflect a difference between the quadriceps and retrodorsolateral nucleus motor populations, Ottem *et al.* (12) assessed BDNF protein at only 14 d after castration, whereas we assessed BDNF at 28 d after castration. Thus, it is possible that changes in BDNF take longer in less androgen-sensitive motoneurons. Alternatively, Ottem *et al.* (12) assessed

BDNF in motoneuron dendrites, whereas we examined motoneuron somata, and thus there could be differences in BDNF across cellular structures.

Treatment with testosterone was effective in reversing or preventing changes in BDNF immunolabeling in both quadriceps and SNB motoneurons, because the frequencies of intensely, moderately, or lightly BDNF-immunolabeled somata in testosterone-treated castrated males did not differ from those of normal males. These results demonstrate that despite the lack of changes in quadriceps motoneuron soma size or muscle weight after castration, some features of the quadriceps neuromuscular system are in fact sensitive to androgens, similar to what is observed for trkB receptors in quadriceps motoneurons (11). Thus, these data suggest a general ability of androgens to regulate BDNF in spinal motoneurons.

Skeletal muscle also expresses BDNF mRNA and protein (18–20, 32), and BDNF is retrogradely transported from the periphery to spinal motoneurons (32, 33). Thus, the decrease in BDNF immunolabeling we observed in quadriceps and SNB motoneurons after castration could potentially reflect changes in the peripheral production of BDNF. Although it is not known whether expression of BDNF mRNA in skeletal muscle is regulated by androgens, BDNF mRNA expression is decreased in skeletal muscles in aged rats (34). Because aged rats are known to have decreased levels of circulating androgens (35, 36), this decrease in BDNF is consistent with regulation by androgens. Indeed, in the present study, BDNF protein concentrations in the VL and BC muscles were maintained at normal levels in castrated males treated with testosterone.

However, although castration reduced BDNF concentrations in the VL muscle, it increased BDNF concentrations in the BC muscle. Opposite effects of castration on BDNF in peripheral tissues has been observed previously; castration decreases BDNF in the vas deferens but increases BDNF in the vesicular and prostate glands of rats (21). The cause of these differential effects is unclear. One possibility is that the postcastration increase in BDNF protein concentration in the BC muscle may reflect changes in the retrograde transport of BDNF to the SNB motoneurons. The retrograde transport of BDNF is activity dependent (37), and castration decreases activity in the SNB neuromuscular system (38, 39). Thus, this decrease in activity could potentially result in a decrease retrograde transport and a concomitant accumulation of BDNF in the BC muscle. Consistent with this idea, after ligation of the sciatic nerve, accumulation of BDNF is observed in the nerve distal to the ligation (40). In addition, activity-dependent retrograde transport of BDNF involves the binding of BDNF to trkB receptors to form a receptor-ligand complex, which is then rapidly transported in vesicles to the cell body (37, 41). After castration, trkB protein is down-regulated in SNB motoneurons (11), and if trkB is similarly down-regulated in SNB axons, it could also account for a decrease in the retrograde transport of BDNF. Testosterone treatment of castrated males maintains activity (42), axonal transport times (43), and trkB protein expression (11) in spinal motoneurons. If retrograde transport is supported in testosterone-treated castrated males, it could account for the normal levels of BDNF protein concentration in the BC muscle. Additionally, if a source of BDNF in SNB somata is indeed the target musculature, the decreases in BDNF immunolabeling after castration and maintenance of BDNF with testosterone we observed are consistent with the idea that retrograde transport of BDNF is diminished in the SNB neuromuscular system of castrated males and restored or prevented with testosterone treatment. There is no evidence that activity in the quadriceps system is altered by castration, potentially explaining the differences we observed in BDNF levels in the muscles of castrates across the two systems. Further studies will be needed to address the effects of gonadal steroids on the expression and retrograde transport of BDNF in the quadriceps and SNB systems.

Interestingly, in normal males, baseline BDNF protein concentrations in the BC were 75% smaller when compared with the VL. Previous studies have reported differences in BDNF concentrations between various somatic muscles. For example, BDNF concentrations were 31% larger in the soleus muscle when compared with the extensor digitorum longus muscle (19). The reason for the baseline differences in BDNF concentrations remains unclear; however, they may reflect differences in the overall activity of the muscles (19).

BDNF expression is regulated through a calcium-dependent signaling pathway, involving the phosphorylation of the cAMP response element (CRE) and its binding protein CREB (44–46). Testosterone has been shown to activate both CRE and CREB (47, 48), and thus, it is possible that the changes in BDNF immunolabeling and protein concentration that we observed in both quadriceps and SNB neuromuscular systems could involve an androgen-mediated regulation of the cAMP signaling pathway for BDNF. Alternatively, many of the actions of testosterone occur through its conversion to dihydrotestosterone or estrogenic metabolites (49), and thus it is possible that the effects we observed on BDNF immunolabeling or protein concentration could be either androgenic or estrogenic in nature. Estrogenic regulation of BDNF has been reported previously in several brain regions (15, 16, 50–53), and although there is no evidence for estrogen accumulation by quadriceps or SNB motoneurons (8), the target muscles of both populations bind estrogens as well as

androgens (54). Subsequent studies with estrogens or nonaromatizable androgens can address this question.

Conclusion

The present results demonstrate that in both highly androgen-sensitive and more typical somatic neuromuscular systems, BDNF protein expression is regulated by gonadal steroids. Thus, in addition to the androgenic regulation of trkB previously reported (11, 12), these data provide another potential mechanism for the interactive effect of BDNF and testosterone previously observed in the SNB (10, 55). More importantly, by examining both the motoneurons and the muscles they innervate, these results demonstrate that within a neural system, BDNF levels in different components are differentially affected by androgen manipulation.

The androgenic regulation of trophic factors has potentially important implications not only for adult maintenance of motoneuron morphology and function but also for neurotherapeutic or protective actions after motoneuron injury or disease (6). It has been suggested that testosterone influences motoneuron morphology by acting at the target musculature to regulate target-derived neurotrophic signals critical for the maintenance of motoneuron morphology (56). Our work implicates BDNF as such a candidate neurotrophic signal (10), and its production, axonal transport, and androgenic regulation could be relevant for a variety of injury paradigms. For example, treatment with testosterone is protective in surviving motoneurons after partial depletion (57, 58), perhaps through a regulation of BDNF in the motoneurons and/or their target musculature. As cited above, testosterone and BDNF are critical in maintaining motoneuron morphology after nerve injury, and the effectiveness of application of BDNF to the cut nerve is consistent with a peripheral source and the importance of axonal transport (10). Finally, the therapeutic effects of gonadal steroids and trophic factors may not only apply to several injury paradigms but as shown by the present study may also apply to all spinal motoneurons, regardless of their degree of androgen sensitivity.

Acknowledgments

We thank James Byers for technical assistance and Dr. Kathryn Lenz and Dr. Cara Wellman for helpful comments on this manuscript.

Address all correspondence and requests for reprints to: Dr. D.R. Sengelaub, Program in Neuroscience, Department of Psychological and Brain Sciences, Indiana University, Bloomington, Indiana 47405. E-mail: sengelau@indiana.edu.

This work was supported by NIH-NINDS NS047264 to D.R.S.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Henderson V, Reynolds D** 2002 Protective effects of estrogen on aging and damaged neural systems. In: Rubin RT, ed. Hormones, brain, and behavior. San Diego: Academic Press; 821–837
2. **Jones KJ** 1993 Gonadal steroids and neural regeneration. A therapeutic role. Adv Neurol 59:227–240
3. **Jones KJ, Brown TJ, Damaser M** 2001 Neuroprotective effects of gonadal steroids on regenerating peripheral motoneurons. Brain Res Rev 37:372–382
4. **Pike CJ** 2001 Testosterone attenuates β-amyloid toxicity in cultured hippocampal neurons. Brain Res 919:160–165
5. **Forgie ML, Kolb B** 2003 Manipulation of gonadal hormones in neonatal rats alters the morphological response of cortical neurons to brain injury in adulthood. Behav Neurosci 117:257–262
6. **Fargo KN, Foecking EM, Jones KJ, Sengelaub DR** 2009 Neuroprotective actions of androgens on motoneurons. Front Neuroendocrinol 30:130–141
7. **Kishino A, Ishige Y, Tatsuno T, Nakayama C, Noguchi H** 1997 BDNF prevents and reverses adult rat motor neuron degeneration and induces axonal outgrowth. Exp Neurol 144:273–286
8. **Breedlove SM, Arnold AP** 1980 Hormone accumulation in a sexually dimorphic motor nucleus of the rat spinal cord. Science 210: 564–566
9. **Yang LY, Arnold AP** 2000 BDNF regulation of androgen receptor expression in axotomized SNB motoneurons of adult male rats. Brain Res 852:127–139
10. **Yang LY, Verhovshek T, Sengelaub DR** 2004 Brain-derived neurotrophic factor and androgen interact in the maintenance of dendritic morphology in a sexually dimorphic rat spinal nucleus. Endocrinology 145:161–168
11. **Osborne MC, Verhovshek T, Sengelaub DR** 2007 Androgen regulates trkB immunolabeling in spinal motoneurons. J Neurosci Res 85:303–309
12. **Ottem EN, Beck LA, Jordan CL, Breedlove SM** 2007 Androgen-dependent regulation of brain-derived neurotrophic factor and tyrosine kinase B in the sexually dimorphic spinal nucleus of the bulbocavernosus. Endocrinology 148:3655–3665
13. **Johnson H, Hökfelt T, Ulfhake B** 1999 Expression of $P75^{ntr}$, trkB and trkC in nonmanipulated and axotomized motoneurons of aged rats. Mol Brain Res 69:21–34
14. **Al-Majed AA, Brushart TM, Gordon T** 2000 Electrical stimulation accelerates and increases expression of BDNF and trkB mRNA in regenerating rat femoral motoneurons. Eur J Neurosci 12:4381–4390
15. **Sohrabji F, Miranda RC, Toran-Allerand CD** 1995 Identification of a putative estrogen response element in the gene coding for BDNF. Proc Natl Acad Sci USA 92:11110–11114
16. **Jezierski MK, Sohrabji F** 2001 Neurotrophin expression in the reproductively senescent forebrain is refractory to estrogen stimulation. Neurobiol Aging 22:309–319
17. **Solum DT, Handa RJ** 2002 Estrogen regulates the development of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus. J Neurosci 22:2650–2659
18. **Küst BM, Copray JC, Brouwer N, Troost D, Boddeke HW** 2002 Elevated levels of neurotrophins in human biceps brachii tissue of amyotrophic lateral sclerosis. Exp Neurol 177:419–427
19. **Mousavi K, Parry DJ, Jasmin BJ** 2004 BDNF rescues myosin heavy chain IIB muscle fibers after neonatal nerve injury. American J Physiol Cell Physiol 287:C22–C29
20. **Arnold A, Yang LY,** The bulbocavernosus and levator ani (BC/LA) muscle complex expresses BDNF protein. Society for Neuroscience 29th Annual Meeting, Miami Beach, FL, 1999, p1269 (Abstract P507.6)
21. **Mirabella N, Squillacioti C, Paone I, Ciarcia R, Russo M, Paino G** 2006 Effects of castration on the expression of brain-derived neurotrophic factor (BDNF) in the vas deferens and male accessory genital glands of the rat. Cell Tissue Res 323:513–522

22. **Smith E, Damassa D, Davidson J** 1977 Hormone administration: peripheral and intracranial implants. New York: Academic Press
23. **Nicolopoulus-Stournaras S, Iles JF** 1983 Motor neuron columns in the lumbar spinal cord of the rat. J Comp Neurol 217:75–85
24. **Kurz EM, Sengelaub DR, Arnold AP** 1986 Androgens regulate the dendritic length of mammalian motoneurons in adulthood. Science 232:395–398
25. **Mesulam NM** 1982 Tracing neural connections with horseradish peroxidase. Chichester, NY: John Wiley and Sons
26. **Dreyfus CF, Dai X, Lercher LD, Racey BR, Friedman WJ, Black IB** 1999 Expression of neurotrophins in the adult spinal cord in vivo. J Neurosci Res 56:1–7
27. **Scarisbrick IA, Isackson PJ, Windebank AJ** 1999 Differential expression of brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5 in adult rat spinal cord: regulation by the glutamate receptor agonist kainic acid. J Neurosci 18:7757–7769
28. **Seevaratnam R, Patel BP, Hamadeh MJ** 2009 Comparison of total protein concentration in skeletal muscle as measured by the Bradford and Lowry assays. J Biochem 145:791–797
29. **Breedlove SM, Arnold AP** 1981 Sexually dimorphic motor nucleus in the rat lumbar spinal cord: response to adult hormonal manipulation, absence in androgen-insensitive rats. Brain Res 225:297–307
30. **Squillacioti C, De Luca A, Paino G, Mirabella N** 2008 Effects of castration on the immunoreactivity to NGF, BDNF and their receptors in the pelvic ganglia of the male rat. Eur J Histochem 52:101–106
31. **Kobayashi NR, Bedard AM, Maxwell TH, Tetzlaff W** 1996 Increased expression of BDNF and trkB mRNA in rat facial motoneurons after axotomy. Eur J Neurosci 8:1018–1029
32. **Koliatsos VE, Clatterbuck RE, Winslow JW, Cayouette MH, Price DL** 1993 Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons in vivo. Neuron 10:359–367
33. **DiStefano PS, Friedman B, Radziejewski C, Alexander C, Boland P, Schick CM, Lindsay RM, Wiegand SJ** 1992 The neurotrophins BDNF, NT-3, and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. Neuron 8:983–993
34. **Ming Y, Bergman E, Edström E, Ulfhake B** 1999 Reciprocal changes in the expression of neurotrophin mRNAs in target tissues and peripheral nerves of aged rats. Neurosci Lett 273:187–190
35. **Ghanadian R, Lewis JG, Chisholm JD** 1975 Serum testosterone and dihydrotestosterone changes with age in rat. Steroids 25:753–762
36. **Kaler LW, Neaves WB** 1981 The androgen status of aging male rats. Endocrinology 108:712–719
37. **Watson FL, Heerssen HM, Moheban DB, Lin MZ, Sauvageot CM, Bhattacharyya A, Pomeroy SL, Segal RA** 1999 Rapid nuclear responses to target-derived neurotrophins require retrograde transport of ligand-receptor complex. J Neurosci 19:7889–7900
38. **Fargo KN, Foster AM, Harty MW, Sengelaub DR** 2003 Estrogen alters the excitability but not morphology of a sexually dimorphic neuromuscular system in adult rats. J Neurobiol 56:66–77
39. **Holmes GM, Sachs BD** 1992 Erectile function and bulbospongiosus EMG activity in estrogen-maintained castrated rats vary with behavioral context. Horm Behav 26:406–419
40. **Tonra JR, Curtis R, Wong V, Cliffer KD, Park JS, Timmes A, Nguyen T, Lindsay RM, Acheson A, DiStefano PS** 1998 Axotomy up-regulates the anterograde transport and expression of brain-derived neurotrophic factor by sensory neurons. J Neurosci 18:4374–4383
41. **Bhattacharyya A, Watson FL, Pomeroy SL, Zhang YZ, Stiles CD, Segal RA** 2002 High-resolution imaging demonstrates dynein-based vesicular transport of activated trk receptors. J Neurobiol 51:302–312
42. **Harty M, Sengelaub DR, Eddins A** 1997 Testosterone alters recruitment in an androgen-sensitive neuromuscular system. Soc Neurosci Abstr 23:1041
43. **Frolkis V, Tanin S, Marcinko V, Muradian K** 1990 Effects of hormones on the fast axoplasmic transport of substances in ventral horns of the spinal cord in rats of different ages. Arch Gerentol 11:33–41
44. **Shieh PB, Hu SC, Bobb K, Timmusk T, Ghosh A** 1998 Identification of a signaling pathway involved in calcium regulation of BDNF expression. Neuron 20:727–740
45. **Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME** 1998 Ca^{2+} influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron 20:709–726
46. **Tao X, West AE, Chen WG, Corfas G, Greenberg ME** 2002 A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF. Neuron 33:383–395
47. **Auger AP, Hexter DP, McCarthy MM** 2001 Sex differences in the phosphorylation of cAMP response element binding protein (CREB) in neonatal rat brain. Brain Res 890:110–117
48. **Aarnisalo P, Palvimo JJ, Jänne OA** 1998 CREB-binding protein in androgen receptor-mediated signaling. Proc Natl Acad Sci USA 95:2122–2127
49. **Hutchison JB** 1997 Gender-specific steroid metabolism in neural differentiation. Cell Mol Neurobiol 17:603–626
50. **Singh M, Meyer E, Simpkins J** 1995 The effect of ovariectomy and estradiol replacement on brain-derived neurotrophic factor messenger ribonucleic acid expression in cortical and hippocampal brain regions of female sprague-dawley rats. Endocrinology 136:2320–2324
51. **Blurton-Jones M, Kuan PN, Tuszynski MH** 2004 Anatomical evidence for transsynaptic influences of estrogen on brain-derived neurotrophic factor expression. J Comp Neurol 468:347–360
52. **Gibbs RB** 1999 Treatment with estrogen and progesterone affects relative levels of brain-derived neurotrophic factor mRNA and protein in different regions of the adult brain. Brain Res 844:20–27
53. **Liu Y, Fowler CD, Young LJ, Yan Q, Insel TR, Wang Z** 2001 Expression and estrogenic regulation of brain-derived neurotrophic factor gene and protein in the forebrain of female prairie voles. J Comp Neurol 433:499–514
54. **Dubé JY, Lesage R, Tremblay RR** 1976 Androgen and estrogen binding in rat skeletal and perineal muscles. Can J Biochem 54:50–55
55. **Yang LY, Arnold AP** 2000 Interaction of BDNF and testosterone in the regulation of adult perineal motoneurons. J Neurobiol 44:308–319
56. **Rand MN, Breedlove SM** 1995 Androgens alters the dendritic arbors of SNB motoneurons by acting upon their target muscles. J Neurosci 15:4408–4416
57. **Fargo KN, Sengelaub DR** 2004 Exogenous testosterone prevents motoneuron atrophy induced by contralateral motoneuron depletion. J Neurobiol 60:348–359
58. **Little CM, Coons KD, Sengelaub DR** 2009 Neuroprotective effects of testosterone on morphology and function of somatic motoneurons following the death of neighboring motoneurons. J Comp Neurol 512:359–372