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Androgenic, But Not Estrogenic, Protection of Motoneurons from Somal and Dendritic Atrophy Induced by the Death of Neighboring Motoneurons

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Abstract

Motoneuron loss is a significant medical problem, capable of causing severe movement disorders or even death. We have been investigating the effects of motoneuron loss on surviving motoneurons in a lumbar motor nucleus, the spinal nucleus of the bulbocavernosus (SNB). SNB motoneurons undergo marked dendritic and somal atrophy following the experimentally induced death of other nearby SNB motoneurons. However, treatment with testosterone at the time of lesioning attenuates this atrophy. Because testosterone can be metabolized into the estrogen estradiol (as well as other physiologically active steroid hormones), it was unknown whether the protective effect of testosterone was an androgen effect, an estrogen effect, or both. In the present experiment, we used a retrogradely transported neurotoxin to kill the majority of SNB motoneurons on one side of the spinal cord only in adult male rats. Some animals were also treated with either testosterone, the androgen dihydrotestosterone (which cannot be converted into estradiol), or the estrogen estradiol. As seen previously, partial motoneuron loss led to reductions in soma area and in dendritic length and extent in surviving motoneurons. Testosterone and dihydrotestosterone attenuated these reductions, but estradiol had no protective effect. These results indicate that the neuroprotective effect of testosterone on the morphology of SNB motoneurons following partial motoneuron depletion is an androgen effect rather than an estrogen effect.

Keywords

steroids; neuroprotection; morphology; dendrites

INTRODUCTION

Motoneuron loss is a significant medical problem, and can result from disease processes or from traumatic injuries. For example, amyotrophic lateral sclerosis (ALS) is a fatal disease characterized by the rapidly progressive loss of motoneurons, affecting 4–8 people per 100,000 population per year, with approximately 5000 new diagnoses made every year in the United States alone (NIH National Library of Medicine, 2006). While ALS is the most prevalent, other diseases including the motor neuron diseases and spinal muscular atrophies (SMAs) are also characterized by progressive loss of motoneurons. Traumatic spinal cord injuries are even more prevalent: in the United States, more than 10,000 people per year survive a spinal cord injury.

Of these, about 45% suffer from spinal motoneuron lesions, and this number rises to about 95% for those with lumbar or sacral injuries (Doherty et al., 2002).

We have begun to examine the effects of motoneuron loss on the structure and function of surviving motoneurons using a rat model of motoneuron death. Our previous studies have demonstrated that surviving motoneurons respond to the loss of their neighbors with marked somal and dendritic atrophy (Fargo and Sengelaub, 2004a,b). This secondary atrophy is responsible for at least some of the movement deficits that accompany degenerative movement disorders and spinal cord trauma. Indeed, work from our laboratory indicates that this atrophy reduces the excitability of the affected motoneuron populations (unpublished observations). Given that we currently lack the technology to replace dead motoneurons, developing the ability to protect surviving motoneurons from secondary atrophy is an important goal.

Gonadal steroids exhibit a wide array of neuroprotective and neurotherapeutic effects (Jones, 1993; Jones et al., 2001; Henderson and Reynolds, 2002; Woolley and Cohen, 2002). For example, testosterone treatment accelerates both axon regeneration and functional recovery following axotomy of spinal or cranial motoneurons (Jones et al., 2001). After crush axotomy of hamster facial motoneurons, treatment with exogenous testosterone accelerates both the rate of axon regeneration (Kujawa et al., 1991) as well as the recovery of motor function (Kujawa et al., 1989; Kujawa and Jones, 1990). Estrogens also exert neurotherapeutic effects. For example, estradiol controls dendritic spine density in the hippocampus (Woolley and McEwen, 1994) and prevents axotomy-induced motoneuron death (Huppenbauer et al., 2005).

Using our model of motoneuron loss, we have begun to examine the neuroprotective effects of gonadal steroids on the secondary atrophy induced in nearby surviving motoneurons. Our previous experiments have demonstrated that testosterone is indeed neuroprotective following motoneuron loss. Treatment with exogenous testosterone attenuates the somal and dendritic atrophy, as well as the attenuated excitability, induced by motoneuron loss (Fargo and Sengelaub, 2004a,b, 2005). However, while testosterone can act directly by activating androgen receptors, it can also act through its conversion to other steroid hormones. For example, testosterone is converted by the enzyme 5 α -reductase to dihydrotestosterone (DHT), which also activates androgen receptors. Testosterone is also converted by the enzyme aromatase to estradiol, which activates estrogen receptors. Because both androgens and estrogens have protective qualities in the nervous system (Henderson and Reynolds, 2002; Woolley and Cohen, 2002; Bialek et al., 2004), it is unclear whether the protective effects on motoneuron morphology following the loss of nearby motoneurons we observed in our previous studies using testosterone were the result of action via androgenic or estrogenic pathways. The purpose of the present experiment was to make this discrimination by treating animals with induced motoneuron loss with either estradiol or DHT, then examining the morphology of surviving nearby motoneurons.

METHODS

Animals

Adult male Sprague Dawley rats (approximately 100 days old; Harlan, Indianapolis, IN) were maintained on a 12:12 h light/dark cycle, with unlimited access to food and water. We used the toxin saporin, conjugated to the cholera toxin B subunit (List Biological Laboratories), to kill motoneurons projecting to the right bulbocavernosus (BC) and levator ani (LA) muscles; these motoneurons have their somata in the spinal nucleus of the bulbocavernosus (SNB), a medial motor nucleus in the lumbosacral spinal cord. The BC and LA muscles surround the base of the penis in male rats; this neuromuscular system is involved in erectile functions and is critically important for male sexual behavior (Sachs, 1982; Hart and Melese-D'Hospital, 1983). The BC/LA complex binds both androgens and estrogens (Dubé et al., 1976); in contrast,

SNB motoneurons accumulate androgens but not estrogens (Breedlove and Arnold, 1980, 1983). The BC/LA muscle complex was exposed under ether anesthesia, and the right BC and LA were each injected with 1 μ L of a 0.1% saporin solution. Saporin is a type I ribosome inactivating protein; it kills cells by irreversibly inactivating ribosomes and thereby halting protein synthesis (Stirpe et al., 1983, 1992). This form of saporin is retrogradely transported from the site of injection, and unilateral injection of saporin into the BC/LA muscle complex kills approximately 60% of ipsilateral SNB motoneurons within 3–6 days, while sparing the nearby contralateral SNB motoneurons (Fargo and Sengelaub, 2004b).

Four groups of saporin-injected animals were produced: a group that received no hormone treatment ($n = 8$), and groups that received supplemental testosterone ($n = 8$), DHT ($n = 8$), or estradiol ($n = 7$). All hormone treatments were started coincident with the saporin injection. Testosterone was administered via 45-mm long, subcutaneous, interscapular implants of Silastic tubing filled with testosterone (4-androsten-17 β -ol-3-one; Steraloids); such implants produce plasma titers of testosterone in the high normal physiological range (Smith et al., 1977), and have previously been demonstrated to reduce motoneuron atrophy induced by the death of nearby motoneurons (Fargo and Sengelaub, 2004a,b). DHT was administered in the same fashion with the exception that the implants were filled with DHT (5 α -androstan-17 β -ol-3-one; Steraloids) and were 30 mm long instead of 45 mm long. This length was chosen because DHT implants of this size and smaller have been used with robust results in previously published studies (Meisel et al., 1984; Forger et al., 1992; Monks et al., 2001). DHT exerts its effects through the androgen receptor, and it cannot be aromatized into estradiol; therefore, if DHT has an effect, it will not be attributable to conversion to estradiol (saporin and DHT treatment $n = 8$). Androgen implants were left in place until sacrifice. Estradiol was administered in the form of daily subcutaneous injections of the hormone dissolved in sesame oil. Each injection consisted of 300 μ g estradiol benzoate (1,3,5(10)-estratrien-3, 17 β -diol 3-benzoate; Steraloids) dissolved in 0.15 mL of sesame oil. This dose and route was chosen because we have previously used it to successfully maintain normal levels of electrophysiological activity in SNB motoneurons in castrated males (Fargo et al., 2003; see also Holmes and Sachs, 1992), and is effective in supporting SNB dendritic morphology during development (Goldstein and Sengelaub, 1994; Burke et al., 1997; Hebbeler and Sengelaub, 2003). Daily estradiol injections continued until sacrifice.

Unlike our prior studies (Fargo and Sengelaub, 2004a,b), no animals were castrated in this experiment. Previous experiments (Verhovshek et al., 2000) have demonstrated that estradiol treatment is insufficient to prevent or reverse castration-induced dendritic retraction. Therefore, if the estradiol-treated animals in this experiment had been castrated, it would have been impossible to determine whether any potential dendritic atrophy was caused by the castration or by an inability of estradiol to reduce atrophy induced by the loss of nearby motoneurons. In addition, the fact that this experimental design does not include castration allows us to determine whether androgen treatment is effective in reducing saporin-induced dendritic atrophy in gonadally intact animals.

Consistent with previous experiments (Fargo and Sengelaub, 2004a,b, 2005), animals in all saporin-injected groups were allowed to survive for 28 days following saporin injection. To serve as controls, a group of untreated intact males ($n = 11$) was also produced.

Histochemistry

Horseradish peroxidase conjugated to the cholera toxin B subunit (BHRP; List Biological Laboratories) was used to retrogradely label left side SNB motoneurons, which given the medial location of the nucleus, have somata that are near the motoneurons killed by the saporin injection. BHRP labeling permits population-level quantitative analysis of motoneuron somal and dendritic morphologies (Kurz et al., 1986; Goldstein et al., 1990). Animals were

anesthetized with ether, and the left BC muscle was exposed and injected with 0.5 μ L of a 0.2% solution of BHRP. Two days after BHRP injection, a period that ensures optimal labeling of SNB motoneurons (Kurz et al., 1986; Goldstein et al., 1990), animals were weighed, overdosed with urethane (approximately 0.25 g/100 g body weight), and perfused intracardially with saline followed by cold fixative (1% paraformaldehyde/1.25% glutaraldehyde). The BC/LA muscle complex was removed from each animal, bisected along the midline, and each side was weighed. The lumbar portion of the spinal cord of each animal was removed, postfixed in the same fixative for 5 h, and then transferred to sucrose phosphate buffer (10% w/v, pH 7.4) overnight for cryoprotection. Spinal cords were then embedded in gelatin and frozen-sectioned transversely at 40 μ m; all sections were collected into four alternate series. One series was stained with thionin for use in cell counts. For visualization of BHRP, the three remaining series were immediately reacted using a modified tetramethyl benzidine protocol (Mesulam, 1982), mounted on gelatin-coated slides, and counterstained with thionin. Digital light micrographs were obtained using an MDS 290 digital camera system (Eastman Kodak Company). Brightness and contrast of these images were adjusted in Adobe Photoshop.

Morphometry

Motoneuron Counts—SNB motoneurons are easily recognizable as large, darkly staining, multipolar cells located in a discrete medial nucleus of the lumbar spinal cord. Estimates of the total number of motoneurons were obtained using the optical disector method outlined by Coggeshall (1992) and a procedure similar to that of West and Gundersen (1990). Counts were made at 500 $\times$ under brightfield illumination. Only motoneurons in which there was a clear nucleus and nucleolus were counted. The length of the disector was approximately 25 μ m, which was adequate for visualizing nucleoli in multiple focal planes. Motoneuron nucleoli were counted as they first appeared in focus while focusing through the *z* axis, and nucleoli in the first focal plane (i.e., “tops”) were not counted. For each animal, motoneuron counts were derived from the unreacted series, in sections spaced 160 μ m apart and distributed uniformly through the entire rostrocaudal extent of each nucleus (mean = 16.2 sections). Because the SNB is a small, discrete nucleus, there is no need to divide it into fields for the purpose of cell counting. Instead, within each section, all SNB motoneurons with nucleoli (excluding “tops”) were counted. To correct for the proportion of sections sampled, the cell count for each animal was multiplied by four.

Using similar methods, the number of BHRP-filled motoneurons was assessed in all sections of the reacted series through the entire rostrocaudal extent of the SNB for all animals. Counts of labeled motoneurons in the SNB were made under brightfield illumination, where somata could be visualized and cytoplasmic inclusion of BHRP reaction product confirmed.

Soma Size—The size of SNB motoneuron somata was assessed in at least one set of alternate sections (160 μ m apart) by measuring the cross-sectional area of BHRP-filled motoneurons. Soma areas of an average of 22.5 motoneurons were measured for each animal using a video-based morphometry system (Stereo Investigator; MicroBright-Field) at a final magnification of 780 $\times$. Soma areas within each animal were then averaged for statistical analysis.

Dendritic Length—For each animal, dendritic lengths in a single representative set of alternate sections were measured under darkfield illumination. Beginning with the first section in which BHRP-labeled fibers were present, labeling through the entire rostrocaudal extent of the SNB dendritic field was assessed in every other section (320 μ m apart) in three dimensions using a computer-based morphometry system (Neurolucida; MicroBrightField) at a final magnification of 250 $\times$. Average dendritic length per labeled motoneuron was estimated by summing the measured dendritic lengths of the series of sections, multiplying by two to correct for sampling, then dividing by the total number of labeled motoneurons in that series. This

method does not attempt to assess the actual total dendritic length of labeled motoneurons (Kurz et al., 1991), but has been shown to be a sensitive and reliable indicator of changes in dendritic morphology in normal development (Goldstein et al., 1990, 1993; Goldstein and Sengelaub, 1993), in response to hormonal manipulation (Kurz et al., 1986, 1991; Forger and Breedlove, 1987; Goldstein et al., 1990; Goldstein and Sengelaub, 1993, 1994; Burke et al., 1997, 1999; Hebbeler et al., 2001, 2002; Hebbeler and Sengelaub, 2003), after changes in dendritic interactions (Goldstein et al., 1993) and afferent input (Kalb, 1994; Hebbeler et al., 2002; Hebbeler and Sengelaub, 2003), and after the death of nearby motoneurons (Fargo and Sengelaub, 2004a,b).

Dendritic Distribution—To assess potential redistributions of dendrites across treatment groups, for each animal the composite dendritic arbor created in the length analysis was divided using a set of axes oriented radially around the central canal. These axes divided the spinal cord into twelve bins of 30° each. The portion of each animal's dendritic arbor per labeled motoneuron contained within each location was then determined. This method provides a sensitive measure of dendritic redistribution in response to changes in dendritic interactions (Goldstein et al., 1993) and afferent input (Hebbeler et al., 2002; Hebbeler and Sengelaub, 2003).

Dendritic Extent—The comparability of BHRP labeling across groups was assessed by quantifying both the rostrocaudal and the radial extent of SNB motoneuron dendritic arbors. The rostrocaudal extent of the dendritic arbor was determined by recording the rostrocaudal distance spanned by SNB dendrites for each animal. The maximal radial extent of the arbor in the mediolateral plane was also measured for each animal, using the same radial axes and resultant 30° bins used for the dendritic distribution analysis: For each bin, the linear distance between the central canal and the most distal BHRP-filled process was measured. Radial dendritic extent is independent of overall dendritic length and reflects the maximal linear distance (in the mediolateral plane) of BHRP transport to the most distal dendritic processes.

All procedures were performed in accordance with the Indiana University Animal Care and Use Guidelines. All data were analyzed by analyses of variance (one way or repeated measures as appropriate), followed by *post hoc* analyses using Fisher's least significant difference (LSD).

RESULTS

Muscle Weights

Unilateral injection of saporin into the BC/LA muscle complex resulted in marked atrophy (approximately 27% reduction in weight) of the injected musculature [0.444 ± 0.016 g (Mean $\pm$ SEM) for saporin-injected animals compared to 0.606 ± 0.030 g for normal controls, LSD, $p < 0.001$; overall test for the effect of group on right side muscle weight $F(4, 30) = 49.47$, $p < 0.001$; Fig. 1], and hormone treatment did not prevent this (right side muscle weights were 0.514 ± 0.021 g for saporin-injected animals treated with testosterone, 0.189 ± 0.013 g for saporin-injected animals treated with estradiol, and 0.424 ± 0.024 g for saporin-injected animals treated with DHT; compared to normal controls, LSD, $ps < 0.01$). However, while none of the various hormone treatments completely prevented saporin-induced reductions in muscle weight, they did have differential effects: testosterone attenuated saporin-induced atrophy (the reduction in muscle weight from normal was only approximately 15%), while estradiol led to increased atrophy (approximately 69% reduction in weight from normal; both treatment groups significantly different from untreated saporin-injected animals; LSD, $ps < 0.05$), and DHT had no effect (compared to untreated saporin-injected animals; LSD, ns).

Notably, the effect of saporin injection on BC/LA weight was specific to the injected side of the muscle complex [left side muscle weights were 0.603 ± 0.017 g for saporin-injected animals

and 0.612 ± 0.030 g for normal controls, LSD, ns; overall test for the effect of group on left side muscle weight $F(4, 30) = 50.59$, $p < 0.001$; Fig. 1]. The various hormone treatments had differential effects on left side muscle weights also: Testosterone increased muscle weight by approximately 21% (0.727 ± 0.039 g for saporin-injected animals treated with testosterone; compared to untreated saporin-injected animals, LSD, $p < 0.01$) while estradiol decreased it markedly (by approximately 60%) and DHT decreased it a small amount (approximately 14%; 0.243 ± 0.016 g for saporin-injected animals treated with estradiol, and 0.520 ± 0.019 g for saporin-injected animals treated with DHT; compared to untreated saporin-injected animals, LSD, $ps < 0.05$).

Motoneuron Counts

The number of SNB motoneurons in the right half of the nucleus in normal controls was well within the normal range (108.80 ± 11.34). In contrast, injection of saporin into the right side of the BC/LA muscle complex resulted in the death of over 60% of ipsilateral SNB motoneurons [42.00 ± 5.07 remaining motoneurons for saporin-injected animals; compared to normal controls, LSD, $p < 0.001$; overall test for the effect of group on motoneuron number $F(4, 25) = 17.25$, $p < 0.001$; Fig. 2], and hormone treatment did not prevent this (34.67 ± 6.42 remaining motoneurons for testosterone-treated animals, 32.00 ± 4.73 estradiol-treated animals, and 33.33 ± 8.98 for DHT-treated animals; compared to normal controls, LSD, $ps < 0.001$); motoneuron numbers did not differ between hormone-treated and untreated saporin-injected animals (LSD, ns). Importantly, SNB motoneuron death induced by saporin injection was specific to the ipsilateral side: On the contralateral side, the average number of SNB motoneurons was 92.27 ± 5.19 , and this did not differ between groups [$F(4, 25) = 0.08$, ns], indicating that saporin injection killed right side SNB motoneurons but not the nearby left side motoneurons.

Morphometry

Somata—Following saporin-induced motoneuron death, surviving nearby motoneurons underwent somal atrophy. Soma areas decreased by 18% [$875.44 \pm 44.69 \mu\text{m}^2$ in untreated saporin-injected animals compared to $1068.71 \pm 57.88 \mu\text{m}^2$ for normal controls, LSD, $p < 0.05$; overall test for the effect of group on soma area $F(4, 23) = 4.46$, $p < 0.01$; Fig. 3]. However, treatment with androgens attenuated this atrophy, with soma areas being reduced by 14% in testosterone-treated animals ($921.44 \pm 48.09 \mu\text{m}^2$) and 13% in DHT-treated animals ($934.41 \pm 50.80 \mu\text{m}^2$). Neither were different from normal controls (LSD, ns), but given their intermediate size, they also did not differ from those of untreated saporin-injected animals (LSD, ns). In contrast, treatment with estradiol was completely ineffective in protecting contralateral SNB motoneurons from somal atrophy, with soma sizes reduced by 30% in estradiol-treated animals ($750.49 \pm 58.06 \mu\text{m}^2$). Soma areas in estradiol-treated animals did not differ from those of untreated saporin-injected animals (LSD, ns), but were significantly smaller than those of normal controls (LSD, $p < 0.001$).

Dendrites—Injection of BHRP into the left BC successfully labeled ipsilateral SNB motoneurons in a manner consistent with previous studies (Kurz et al., 1986, 1991; Fargo and Sengelaub, 2004a,b). SNB motoneurons displayed their characteristic multipolar morphologies (Kurz et al., 1986; Sasaki and Arnold, 1991), with dendritic arbors projecting ventrolaterally, dorsomedially, and across the midline into the area of the contralateral SNB (Fig. 4). An average of $50.57 (\pm 3.96)$ motoneurons per animal were labeled with BHRP.

Following saporin-induced motoneuron death, surviving nearby motoneurons underwent marked dendritic atrophy. Dendritic length decreased by 66% ($2589.31 \pm 399.26 \mu\text{m}$ in untreated saporin-injected animals compared to $7694.93 \pm 1728.55 \mu\text{m}$ for normal controls, LSD, $p < 0.01$; overall test for the effect of group on arbor per cell $F(4, 23) = 4.66$, $p < 0.01$;

Fig. 5]. However, treatment with androgens attenuated this dendritic atrophy, with dendritic lengths being reduced by only 32% in testosterone-treated animals ($5234.49 \pm 1195.14 \mu\text{m}$) and only 7% in DHT-treated animals ($7181.43 \pm 1435.71 \mu\text{m}$); neither were different from normal controls (LSD, ns). Dendritic lengths in DHT-treated animals were almost three times larger than those of untreated saporin-injected animals (LSD, $p < 0.01$); similarly, dendritic lengths in testosterone-treated animals were twice as large as those of untreated saporin-injected animals, but this difference failed to reach significance (LSD, ns). In contrast, treatment with estradiol was completely ineffective in protecting surviving SNB motoneurons from dendritic atrophy, with dendritic lengths reduced by 72% in estradiol-treated animals ($2193.08 \pm 384.16 \mu\text{m}$). Dendritic lengths in estradiol-treated animals did not differ from those of untreated saporin-injected animals (LSD, ns), but were significantly smaller than those of normal controls, LSD, $p < 0.01$).

Dendritic length was nonuniform across radial bins, and a repeated-measures ANOVA revealed a significant effect of radial location [$F(11, 253) = 111.39$, $p < 0.001$; Fig. 6]. Consistent with the results of the arbor per cell analysis, there was also a significant effect of group [$F(4, 23) = 4.75$, $p < 0.01$]. Reductions in dendritic length occurred throughout the radial distribution, ranging from 30% (210° – 240°) to 94% (90° – 120°) in untreated saporin-injected animals compared to normal controls (LSD, $p < 0.01$). Treatment with androgens—either testosterone or DHT—attenuated these reductions (compared to normal controls; LSD, ns). Dendritic lengths per bin in testosterone-treated animals were significantly greater than those of untreated saporin-injected animals throughout most of the radial distribution (0° – 240° ; LSD, $p < 0.05$), and were significantly greater at all radial locations in DHT-treated animals (LSD, $p < 0.01$). Consistent with the overall analysis above, dendritic lengths per bin in estradiol-treated animals were reduced throughout the distribution (compared to normal controls; LSD, $p < 0.01$; Fig. 6). Dendritic lengths per bin in estradiol-treated animals did not differ from those of untreated saporin-injected animals (LSD, ns).

Radial dendritic extent (Fig. 7) was nonuniform across bins, and repeated-measures ANOVA revealed significant effects of location [$F(11, 253) = 100.81$, $p < 0.001$] and group [$F(4, 23) = 7.90$, $p < 0.001$] with no significant interaction [$F(44, 253) = 1.16$, ns]. Radial dendritic extents in estradiol-treated animals did not differ from those of untreated saporin-injected animals (LSD, ns), and were decreased in both groups compared to normal controls (LSD, $ps < 0.05$). Treatment with androgens protected surviving SNB motoneurons from decreases in radial dendritic extent; extent measures in DHT-treated animals did not differ from those of normal controls or testosterone-treated animals (LSD, ns). Radial dendritic extents in DHT-treated animals were consistently greater than those of untreated saporin-injected animals at all radial locations, and significantly so at 300° – 120° (LSD, $ps < 0.05$); although they were also consistently longer in testosterone-treated animals, radial dendritic extents were only significantly so at 60° – 120° , and 300° – 360° (LSD, $ps < 0.05$).

Rostrocaudal dendritic extent also varied by group [$F(4, 23) = 3.30$, $p < 0.05$; Fig. 8]. *Post hoc* analyses revealed that this difference was due to significantly greater rostrocaudal extents in both testosterone-treated ($3253.33 \pm 114.39 \mu\text{m}$) and DHT-treated animals ($3200.00 \pm 184.75 \mu\text{m}$) compared to untreated ($2586.67 \pm 167.39 \mu\text{m}$) and estradiol-treated animals ($2624.00 \pm 129.98 \mu\text{m}$; LSD, $ps < 0.04$). However, while rostrocaudal extents were slightly shorter in untreated and estradiol-treated animals (which did not differ from each other; LSD, ns), no group differed significantly from normal controls ($3008.00 \pm 259.97 \mu\text{m}$; LSD, ns).

DISCUSSION

Testosterone treatment protects SNB motoneurons from somal and dendritic atrophy, and from concomitant reductions in excitability, resulting from the death of nearby motoneurons (Fargo

and Sengelaub, 2004a,b, 2005). However, testosterone can act directly as an androgen or be metabolized into other steroid hormones, both androgenic and estrogenic. It was therefore unknown whether the protective effects of testosterone were due to its action in androgenic pathways, or due to its conversion to an estrogen and subsequent action in estrogenic pathways. In the present experiment, we treated animals with induced motoneuron loss either with testosterone or one of its two metabolites, the androgen DHT or the estrogen estradiol. Morphological analysis of surviving nearby SNB motoneurons indicates that androgens, but not estrogen, protect against somal and dendritic atrophy following partial motoneuron loss.

Muscle Weights

Saporin injection decreased the weight of the injected side of the muscle complex, yet had no effect on the weight of the contralateral muscles (Fig. 1). These results are comparable to previous findings (Fargo and Sengelaub, 2004a,b,2005) and indicate that saporin treatments were confined to the ipsilateral musculature.

Testosterone and its metabolites have very different effects on muscle weights (Fig. 1). Testosterone was the most trophic of the three hormones tested—it increased the weight of the noninjected muscle above normal, and partially ameliorated the atrophy induced by saporin in the injected muscle. In contrast, DHT had no beneficial effect on the weight of the saporin-injected muscles, and even resulted in a small but significant *loss* of weight in the noninjected muscles. Given that DHT is an androgen and an anabolic steroid, it would seem unusual for it to actually cause muscle atrophy. In fact, it has previously been reported that DHT maintains BC/LA muscle weight in castrated animals over at least the first 10 days postcastration (Foster and Sengelaub, 2004). However, the muscle weight data from Forger et al. (1992) and unpublished data from our laboratory independently confirm that BC and LA muscle weights are better maintained by testosterone than by DHT over longer periods. Finally, estradiol treatment markedly reduced BC/LA muscle weights. We believe it is unlikely that either estradiol or DHT had direct atrophic effects on the muscle. Rather, treatment with exogenous testosterone, DHT, or estradiol would all be expected to depress the production of endogenous testosterone via negative feedback on the HPG axis (Bermant and Davidson, 1974;Hedge et al., 1987). In the testosterone-treated animals, while endogenous testosterone production might be depressed, the exogenous testosterone supplied via Silastic implants would be enough to maintain, indeed even increase, BC/LA muscle weights; in contrast, in estradiol- or DHT-treated animals, there would not be enough circulating testosterone to maintain normal BC/LA weight.

Motoneuron Counts

Unilateral saporin injection resulted in the death of over 60% of ipsilateral motoneurons (Fig. 2). Furthermore, saporin injection resulted in a laterally specific depletion of SNB motoneurons—motoneuron number in the nearby contralateral SNB did not differ from normal following saporin injection. In some model preparations, steroid hormones prevent injury-induced neuron death (Ahlbom et al., 2001;Hammond et al., 2001;Pike, 2001;Ramsden et al., 2003;Huppenbauer et al., 2005). It was therefore important to establish that saporin injection killed approximately the same number of motoneurons in the untreated and steroid-treated groups. As can be clearly seen in Figure 2, saporin injection was equally effective in killing motoneurons in all saporin-injected groups, regardless of hormone treatment. This means that any potential beneficial effects of steroid treatment on the morphology of nearby surviving motoneurons cannot be attributed to a hormone-mediated attenuation of saporin's ability to kill motoneurons.

Morphometry

Somata—After the loss of motoneurons by saporin injection, cross-sectional area of surviving nearby motoneurons is reduced by 18% (Fig. 3), consistent with the results of our previous studies (Fargo and Sengelaub, 2004a,b). Treatment with either testosterone or DHT attenuates this atrophy, but treatment with estradiol does not. These data indicate that the neuroprotective effect of testosterone treatment on soma size in this model is an androgenic effect, as it can be reproduced by treating with testosterone's androgenic metabolite DHT, but not its estrogenic metabolite estradiol.

Dendrites—The dendritic length results parallel the soma size results. Motoneuron loss by saporin injection is accompanied by the loss of more than 66% of the dendritic arbor in surviving nearby motoneurons (Fig. 5). Treatment with either testosterone or DHT attenuates this loss, but treatment with estradiol does not. Analysis of the radial distribution of dendritic length reveals that dendritic length in surviving motoneurons is lost in every radial bin following saporin injection. Treatment with either testosterone or DHT, but not estradiol, attenuates these decreases again, in every radial bin (Fig. 6). These data indicate that the neuroprotective effect of testosterone treatment on dendritic length in this model, as with soma size, is an androgenic effect.

Dendritic extent displays a pattern similar to overall dendritic length. Following saporin-induced motoneuron loss, radial dendritic extent is decreased across all radial bins in untreated and estradiol-treated animals (Fig. 7). However, treatment with androgens attenuated this decrease in radial extent, and extent measures in animals treated with DHT did not differ from those of normal animals or animals treated with testosterone. Differences in rostrocaudal dendritic display a similar pattern. Although no group differed significantly from normal controls, rostrocaudal extent measures in untreated and estradiol treated animals were slightly smaller than those of animals treated with androgens. These data suggest that androgen treatment exerts a protective effect on dendritic extent as well as dendritic length, while estradiol is ineffective in all of these measures.

Previous studies have demonstrated that neither axonal transport of BHRP (Leslie et al., 1991) nor dendritic transport as demonstrated by the rostrocaudal or radial extent of dendritic labeling (Kurz et al., 1991; Goldstein and Sengelaub, 1994; Hebbeler et al., 2002; Fargo and Sengelaub, 2004b) are affected by hormone levels. Thus, in the present study, we believe that the differences we observed across hormone treatment groups reflect true dendritic atrophy in surviving motoneurons in untreated and estradiol-treated animals, which is attenuated by treatment with androgens. The possibility that confounds arising from saporin injection could affect retrograde transport is also an important consideration, as such an artifact could potentially result in apparent alterations in dendritic morphology. No significant differences in the rostrocaudal extents of SNB dendrites in any group compared to normal values were observed, but radial extents in untreated and estradiol-treated animals were reduced after saporin injection. However, no such effect was observed in either testosterone- or DHT-treated animals, indicating that reductions in extent cannot be due to saporin injection *per se*.

Androgenic Neuroprotection in the SNB

Because both testosterone and DHT were neuroprotective, while estradiol was not, the present results suggest that the neuroprotective effects of steroid treatment in this model are androgenic in nature. This is consistent with what is known about adult morphological plasticity in the SNB system. For example, following castration, treatment with androgens is capable of maintaining adult SNB soma size, dendritic length, and target muscle mass (Wainman and Shipounoff, 1941; Breedlove and Arnold, 1981; Kurz et al., 1986; Forger et al., 1992). In contrast, estradiol treatment in adults fails to prevent castration-induced atrophy of SNB

motoneuron somata, muscle mass (Forger et al., 1992), or dendrites (unpublished observations from our laboratory). Similarly, blockade of estradiol synthesis in intact adult males with the aromatase inhibitor fadrozole has no effect on SNB motoneuron morphology (unpublished observations from our laboratory). Additionally, SNB motoneurons accumulate androgens, but not estradiol (Breedlove and Arnold, 1980, 1983), and express high levels of 5 α -reductase (Pozzi et al., 2003), the enzyme that converts testosterone into DHT. While the lack of a protective effect of estradiol in the current study is consistent with the lack of estrogenic effects in adulthood described above, it is interesting that during development estrogens are capable of supporting SNB dendritic growth (Goldstein and Sengelaub, 1994; Nowacek and Sengelaub, 2006). However, this effect is transient, and estradiol treatment after 4 weeks of age is ineffective in supporting dendritic morphology (Goldstein and Sengelaub, 1994). This transient estrogenic effect on dendritic support may be limited by postnatal changes in the developing spinal cord, for example via the decline in NMDA receptor expression (Verhovshek et al., 2005). Taken together, these observations suggest that adult SNB motoneuron morphology is sensitive to androgens, but not to estradiol. Thus, the SNB stands in contrast to some other areas in the nervous system, where estradiol has powerful neuroprotective effects (Woolley and Cohen, 2002). For example, estradiol protects cortical and hippocampal neurons from cell death induced by ischemia (Hoffman et al., 2006) and promotes cell survival (Huppenbauer et al., 2005) and axonal regeneration (Tanzer and Jones, 1997) in brain stem motoneurons following peripheral axotomy.

The present data do not indicate how androgens might be acting in order to protect motoneuron morphology after the loss of nearby motoneurons. However, androgen effects on SNB motoneurons and their target muscles have been the subject of intense study, and so clues can be gleaned from the literature. Androgens are known to regulate several important biomolecules in the SNB. For example, levels of immunoreactivity for the ciliary neurotrophic factor receptor α (Forger et al., 1998) and BCL-2 (Zup and Forger, 2002) are androgen-dependent in SNB motoneurons, as are levels of mRNA expression for the major cytoskeletal elements β -actin (Matsumoto et al., 1992) and β -tubulin (Matsumoto et al., 1993). Moreover, the SNB target muscles also contain high numbers of androgen receptors (Monks et al., 2004), and androgens regulate a variety of characteristics of these muscles, including fiber size (Venable, 1966), neuromuscular junction size (Bleisch and Harrelson, 1989; Balice-Gordon et al., 1990), acetylcholine receptor number (Bleisch et al., 1982; Bleisch and Harrelson, 1989), muscle excitability (Foster and Sengelaub, 2004), and the number of functional calcium channels at the neuromuscular junction (Nudler et al., 2005). These results raise the possibility that the neuroprotective effects of androgens after saporin-induced motoneuron depletion in this system could be mediated by the muscle (Fargo and Sengelaub, 2004a,b). Rand and Breedlove (1995) showed that testosterone can regulate dendrites in adulthood by acting at the target musculature, suggesting that androgens regulate a neurotrophic signal from the muscle that is critical in the maintenance of dendritic organization (Rand and Breedlove, 1995). Similarly, Yang et al. (2004) demonstrated that brain-derived neurotrophic factor (BDNF) interacts with testosterone in the maintenance of adult SNB dendritic arbors and, by applying BDNF peripherally to cut BC/LA nerves, showed that SNB dendritic morphology can be regulated by trophic substances from the neuromuscular periphery. Alternatively, cell-autonomous effects of androgens in the SNB have been observed wherein only those motoneurons with functional androgen receptors show changes in soma size (Watson et al., 2001) or N-cadherin immunoreactivity (Monks et al., 2001), raising the possibility that the motoneurons themselves could be a site for the neuroprotective effects of androgens after saporin-induced motoneuron depletion.

The current data support the idea that androgens might play a beneficial role in the treatment of spinal cord injuries and motoneuron diseases. Androgens are already known to prevent or reduce motoneuron death in several experimental paradigms (Ahlbom et al., 2001; Hammond

et al., 2001; Pike, 2001; Ramsden et al., 2003; Huppenbauer et al., 2005). In addition, the present results suggest that androgens are also capable of attenuating secondary atrophy in surviving nearby motoneurons. An important question is whether these benefits would persist after a short-term, “pulse” treatment with androgen (see, e.g., Tanzer and Jones, 2004) or require continuous treatment with exogenous androgen.

CONCLUSIONS

Testosterone has already been shown to reduce the somal and dendritic atrophy of motoneurons that accompany the death of nearby motoneurons following saporin injection. However, because testosterone can be converted into estradiol, which also has neuroprotective effects but acts via estrogen receptors instead of androgen receptors, it was important to determine whether the effects of testosterone in this model are androgenic or estrogenic in nature. Because treatment with either testosterone or DHT attenuated atrophy, but treatment with estradiol did not, we have established that the neuroprotective effects of testosterone in this model are androgenic in nature.

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Figure 1.

BC/LA muscle weights of normal controls and saporin-injected animals that were either untreated (SAP), or treated with testosterone (SAP+T), estradiol (SAP+E), or DHT (SAP+DHT). Filled bars represent the BHRP-injected side of the muscle complex. Saporin injection into the contralateral side had no effect on this side of the muscle. Steroid treatments had differential effects. Testosterone increased muscle weight above normal. In contrast, treatment with both estradiol and DHT resulted in reduced muscle weights, with estradiol having a much larger effect than DHT. Open bars represent the saporin-injected side of the muscle complex (for normal animals, the open bars represent the right side of the muscle complex, but received no saporin injection). Saporin injection decreased muscle weight on the injected side. Steroid treatments had differential effects. Testosterone increased muscle weight, but only partially attenuated the effect of saporin injection. Estradiol actually decreased muscle weight even more than saporin injection alone. DHT neither increased nor decreased muscle weight compared to saporin injection alone. Bar heights represent means $\pm$ SEM. * indicates significantly different from normal controls. † indicates significantly different from untreated saporin-injected animals.

Figure 2.

Numbers of thionin-stained SNB motoneurons of normal controls and saporin-injected animals that were either untreated (SAP), or treated with testosterone (SAP+T), estradiol (SAP+E), or DHT (SAP+DHT) for both the BHRP-injected side (filled bars) and the saporin-injected side (open bars). For normal controls, the open bars represent the right half and the filled bars represent the left half of the SNB. Saporin killed more than 60% of the ipsilateral SNB motoneurons, regardless of hormone status, and did not kill contralateral motoneurons. Bar heights represent means $\pm$ SEM. * indicates significantly different from normal controls.

Figure 3.

Cross-sectional soma areas of SNB motoneurons of normal controls and saporin-injected animals that were either untreated (SAP), or treated with testosterone (SAP+T), estradiol (SAP+E), or DHT (SAP+DHT). Following saporin-induced motoneuron death, the average soma area of surviving nearby motoneurons decreased by approximately 18%. Treatment with either testosterone or DHT attenuated this decrease, but treatment with estradiol was ineffective. Bar heights represent means $\pm$ SEM. * indicates significantly different from normal controls.

Figure 4.

Left: Darkfield photomicrographs of transverse sections through the lumbar spinal cords of saporin-injected animals treated with testosterone, estradiol, or DHT, after BHRP injection into the left BC muscle. *Right:* Computer-generated composites of BHRP-labeled somata and processes drawn at 320 μm intervals through the entire rostrocaudal extent of the SNB; these composites were selected because they are representative of their respective group average dendritic lengths.

Figure 5.

Dendritic lengths of SNB motoneurons of normal controls and saporin-injected animals that were either untreated (SAP), or treated with testosterone (SAP+T), estradiol (SAP+E), or DHT (SAP+DHT). Following saporin-induced motoneuron death, surviving nearby motoneurons 66% of their dendritic length. Treatment with either testosterone or DHT attenuated this dendritic atrophy, while treatment with estradiol was ineffective. Bar heights represent means $\pm$ SEM. * indicates significantly different from normal controls. † indicates significantly different from untreated saporin-injected animals.

Figure 6.

Top: Drawing of spinal gray matter divided into radial sectors for measure of SNB dendritic distribution. *Bottom:* Length per radial bin of SNB dendrites of normal controls and saporin-injected animals that were either untreated (SAP), or treated with testosterone (SAP+T), estradiol (SAP+E), or DHT (SAP+DHT). For graphic purposes, dendritic length measures have been collapsed into 6 bins of 60° each. SNB motoneuron dendritic arbors display a non-uniform distribution, with the majority of the arbor located between 180° and 300°. Following saporin-induced motoneuron death, surviving nearby motoneurons had reduced dendritic length in every radial bin. Treatment with testosterone or DHT attenuated this reduction, but treatment with estradiol did not. Bar heights represent means $\pm$ SEM. * indicates significantly different from normal controls. † indicates significantly different from untreated saporin-injected animals.

Figure 7.

Top: Drawing of spinal gray matter divided into radial sectors for measure of SNB radial dendritic extent. *Bottom:* Radial extents of SNB dendrites of normal controls and saporin-injected animals that were either untreated (SAP), or treated with testosterone (SAP+T), estradiol (SAP+E), or DHT (SAP+DHT). For graphic purposes, dendritic extent measures have been collapsed into 6 bins of 60° each. Following saporin-induced motoneuron death, surviving nearby motoneurons in SAP and SAP+E animals had reduced dendritic extent in every radial bin; extent measures in animals treated with DHT did not differ from those of normal animals or animals treated with testosterone. Bar heights represent means $\pm$ SEM. * indicates significantly different from normal controls. † indicates significantly different from untreated saporin-injected animals.

Figure 8. Rostrocaudal extents of SNB dendrites of normal controls and saporin-injected animals that were either untreated (SAP), or treated with testosterone (SAP+T), estradiol (SAP+E), or DHT (SAP+DHT). No group differed significantly from normal controls. Bar heights represent means $\pm$ SEM. † indicates significantly different from untreated saporin-injected animals.