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Neuroprotective Effects of Testosterone on Motoneuron and Muscle Morphology Following Spinal Cord Injury

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Abstract

Treatment with testosterone is neuroprotective/neurotherapeutic after a variety of motoneuron injuries. Here we assessed whether testosterone might have similar beneficial effects after spinal cord injury (SCI). Young adult female rats received either sham or T9 spinal cord contusion injuries and were implanted with blank or testosterone-filled Silastic capsules. Four weeks later, motoneurons innervating the vastus lateralis muscle of the quadriceps were labeled with cholera toxin-conjugated horseradish peroxidase, and dendritic arbors were reconstructed in three dimensions. Soma volume, motoneuron number, lesion volume, and tissue sparing were also assessed, as were muscle weight, fiber cross-sectional area, and motor endplate size and density. Contusion injury resulted in large lesions, with no significant differences in lesion volume, percent total volume of lesion, or spared white or gray matter between SCI groups. SCI with or without testosterone treatment also had no effect on the number or soma volume of quadriceps motoneurons. However, SCI resulted in a decrease in dendritic length of quadriceps motoneurons in untreated animals, and this decrease was completely prevented by treatment with testosterone. Similarly, the vastus lateralis muscle weights and fiber cross-sectional areas of untreated SCI animals were smaller than those of sham-surgery controls, and these reductions were both prevented by testosterone treatment. No effects on motor endplate area or density were observed across treatment groups. These findings suggest that regressive changes in motoneuron and muscle morphology seen after SCI can be prevented by testosterone treatment, further supporting a role for testosterone as a neurotherapeutic agent in the injured nervous system.

INDEXING TERMS

steroids; neuroprotection; morphology; dendrites

In the United States, more than 10,000 people per year survive a spinal cord injury (SCI); 45% suffer from spinal motoneuron lesions, and this number rises to 95% for those with lumbar or sacral injuries (Doherty et al., 2002). It is estimated that 200,000 people in the United States are living with a disability caused by an SCI (National Center for Injury Prevention and Control, 2002).

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The pathophysiology of SCI involves both immediate and secondary effects. After the initial mechanical deformation, a protracted period of progressive damage occurs, causing spreading of the lesion and further segmental destruction. A variety of mechanisms contribute to this progressive secondary injury, including excitotoxicity (Liu et al., 1991), free radical generation (Diaz-Ruiz et al., 2002), protease activation (Wang et al., 1997), and inflammation (Ritz and Hausmann, 2008; Liu et al., 2009; Liu and Xu, 2010), resulting in the death of motoneurons, interneurons, and glial cells in the spinal cord (Liu et al., 1997; National Institute of Neurological Disorders and Stroke, 2005; Liu and Xu, 2010). Similarly, damage to spinal nerves resulting in laceration and avulsion of spinal roots (e.g., cauda equina injury with high-impact motor vehicle accidents, Moschilla et al., 2001) can lead to the death of motoneurons and preganglionic autonomic neurons in the spinal cord, resulting in autonomic and motor dysfunction (Hoang et al., 2003).

Death is not the only outcome for injured spinal motoneurons, and importantly, the remaining motoneurons after such insults show a variety of morphological and functional changes. For example, denervation of motoneurons can result in dendritic reorganization (Hebbeler and Sengelaub, 2003) or atrophy (Standler and Bernstein, 1984; Gazula et al., 2004). Similarly, after peripheral axotomy, motoneurons show functional and biochemical changes (Titmus and Faber, 1990; Bisby and Tetzlaff, 1992), as well as dendritic atrophy (Sumner and Watson, 1971; Brännström et al., 1992; O'Hanlon and Lowrie, 1995).

Gonadal steroid hormones provide protection from many of the pathophysiological changes seen after SCI, for example, by reducing the inflammation and free radical generation that contribute to progressive secondary injury. Following SCI, female mice show better recovery than males (Hauben et al., 2002; Farooque et al., 2006), an effect thought to be due to the neuroprotective effects of estrogens. Consistent with this hypothesis, after SCI, treatment of male rats with estradiol resulted in improved motor function, reduced lesion size, increased white matter sparing, and earlier cytokine release and astroglial response (Yune et al., 2004; Ritz and Hausmann, 2008; Kachadroka et al., 2010).

Treatment with testosterone also produces a wide array of neuroprotective and neurotherapeutic effects (Fargo et al., 2009a). For example, testosterone is neuroprotective from oxidative stress (Ahlbom et al., 2001) and protects against cell death (Pike, 2001). Testosterone accelerates both axon regeneration and functional recovery following axotomy (Jones et al., 2001). Testosterone treatment attenuates the synaptic stripping seen after motoneuron injury, preserving central input to the motoneurons (Jones et al., 1997a). Similarly, neuronal death induces dendritic atrophy and a concomitant deficit in excitability in spinal motoneurons, and treatment with testosterone attenuates these regressive changes in morphology and function (Fargo and Sengelaub, 2004a,b, 2007; Fargo et al., 2009b; Little et al., 2009; Wilson et al., 2009). Indeed, treatment with testosterone improves motor function in SCI patients. Patients treated with testosterone had higher American Spinal Injury Association (ASIA) discharge motor scores, a result ascribed to either improved strength through the anabolic effects of testosterone on skeletal muscle or its neuroprotective/neuroregenerative effects (Clark et al., 2008).

Given the diverse neuroprotective effects of testosterone (Fargo et al., 2009a), many of which are directly relevant to the pathophysiology seen after SCI (e.g., free radical generation, cell death, deafferentation, synaptic stripping, axotomy, and resultant loss of motor function), and the positive results on motor function reported in patients treated with testosterone after SCI (Clark et al., 2008), we hypothesized that testosterone would have neuroprotective/neurotherapeutic effects on spinal motoneurons and their target musculature after SCI.

MATERIALS AND METHODS

Young adult female rats (Sprague-Dawley, Harlan), approximately 12 weeks old, were maintained on a 12:12-hour light/dark cycle with food and water freely available. All surgical interventions and postoperative animal care were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council) and were approved by the Indiana University Institutional Animal Care and Use Committee.

Spinal cord contusion and hormone treatment

Rats were anesthetized by injection of sodium pentobarbitol (Nembutal; 45 mg/kg, i.p.; Abbott Laboratories, Chicago, IL). A T9 laminectomy was performed to expose the underlying thoracic spinal cord segment(s), and animals received a severe (a 10-g weight dropped from a height of 25 mm) contusion injury by using an NYU impactor. SCI at the T9 vertebra (T9–11 spinal cord) was intended to preserve the central pattern generators at L1–2 required for locomotor function, and the relevant motoneurons for our analysis. The muscle and skin were then closed in layers by using 5-0 sutures and stainless steel wound clips, respectively. Postoperative care followed previous protocols (Bamber et al., 2001; Xu et al., 1999).

Immediately following contusion injury, rats were implanted with subcutaneous Silastic capsules (3.18 mm O.D., 1.57 mm I.D., 45 mm long) that were either empty (n = 6) or filled with testosterone (4-androsten-17β-ol-3-one; Steraloids, Newport, RI; n = 5). Such implants produce plasma titers of testosterone in the normal physiological range for male rats, and maintain stable levels for durations similar to those used in the current study (Smith et al., 1977), and have previously been demonstrated to attenuate motoneuron atrophy and concomitant deficits in activation after induced motoneuron death in both male (Fargo and Sengelaub, 2004a,b, 2007; Fargo et al., 2009b; Little et al., 2009) and female (Wilson et al., 2009) rats. Animals were allowed to survive for 4 weeks following SCI, a length of time sufficient to observe induced effects on motoneuron morphology (Fargo and Sengelaub, 2004a,b, 2007; Little et al., 2009; Wilson et al., 2009). An additional group of age-matched, sham-injured females served as normal controls (n = 7).

Histological and histochemical processing

Four weeks after injury, animals were reanesthetized, and the left vastus lateralis muscle of the quadriceps was exposed and injected with horseradish peroxidase conjugated to the cholera toxin B subunit (BHRP; 2 μl, 0.2%; List Biological, Campbell, CA). BHRP labeling permits population-level quantitative analysis of motoneuron somal and dendritic morphologies (Goldstein et al., 1990; Kurz et al., 1986). Forty-eight hours after BHRP injection, a period that ensures optimal labeling of motoneurons (Gold-stein et al., 1990; Kurz et al., 1986), animals were weighed and received a lethal dose of Nembutal (60 mg/kg, i.p.), and were then perfused intracardially with saline followed by cold fixative (1% paraformaldehyde/1.25% glutaraldehyde).

Lesion reconstruction

To assess potential effects on lesion volume and tissue sparing, spinal cords were carefully dissected to preserve the surrounding dura mater, and a 15-mm thoracic spinal cord segment including the lesion was removed, postfixed overnight in the same fixative as used for perfusion, and transferred to sucrose phosphate buffer (30% w/v, pH 7.4). Thoracic segments were then embedded in gelatin, frozen, and sectioned transversely at 40-μm; alternate sections were collected into two series, and mounted on gelatin-coated slides. One series was stained for myelin by using Luxol fast blue, and the other series was stained with cresyl violet and eosin for assessing lesion and spared tissue volume as described previously

(Iannotti et al., 2004). The cross-sectional areas of lesion or spared white and gray matter for each animal were measured in sections located 400 μm apart and spanning the entire rostrocaudal extent of the lesion by using a video-based morphometry system (Stereo Investigator; MBF Bioscience, Williston, VT) at a final magnification of 202×.

An unbiased estimation of the percentage of spared tissue was calculated by using the Cavalieri method (Michel and Cruz-Orive, 1988). The total volume of spared white and gray matter was calculated by summing their individual subvolumes (Oorschot, 1994). Individual subvolumes of spared tissue were calculated by multiplying the cross-sectional area A × d, where d represents the distance between sections (400 μm). Septae or fibrous bands of tissue observed within and/or spanning areas of cystic cavitation were not considered to represent spared tissue. The percent total volume of spared white and gray matter was calculated by dividing the total volume of spared white and gray matter by the total tissue volume of the corresponding region (×100), respectively. Estimation of total and percent total lesion volume (which included areas of cavitation and fibrosis) was determined using identical procedures.

Motoneuron number and morphology

The vastus lateralis muscle is innervated by motoneurons located in column 3 of the lateral motor column in the L2 spinal segment (Nicolopoulos-Stournaras and Iles, 1983; Brushart and Seiler, 1987; Al-Majed et al., 2000). Following perfusion, the lumbar portion of the spinal cord of each animal was removed, postfixed for 5 hours in the same fixative as used for perfusion, and then transferred to sucrose phosphate buffer (10% w/v, pH 7.4) overnight for cryoprotection. Spinal cords were then embedded in gelatin, frozen, and sectioned transversely at 40 μm; 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 by using a modified tetramethyl benzidine protocol (Mesulam, 1982), mounted on gelatin-coated slides, and counterstained with thionin.

Motoneuron counts

To assess potential effects on motoneuron loss after SCI, counts of motoneurons in the quadriceps motor pool were performed. Motoneurons innervating the quadriceps muscles do not form a discrete nucleus, but instead are contained within the large continuous populations of motoneurons located within the lateral motor column. Thus, to identify the appropriate area within the lateral motor column for motoneuron counts in the unreacted series, we used a method similar to that of Little et al. (2009).

Briefly, for each animal the range of sections in which motoneurons labeled with BHRP after injection into the vastus lateralis muscle were present in the reacted series was identified, and then motoneuron counts were performed in the appropriate matching sections in the unreacted series. For each animal, estimates of the total number of motoneurons in the left and right lateral motor columns were obtained by using the optical disector method as previously described (Little et al., 2009). Counts were made at 937.5× under brightfield illumination. Motoneurons are easily recognizable as large, darkly staining, multipolar cells.

A counting frame (110 × 80μm) was moved systematically throughout an area of each ventral horn (approximately 500 × 500 μm, defined by the actual distribution of BHRP-labeled somata from all of the animals used in the study) in each section within the identified range. Only motoneurons in which there was a clear nucleus and nucleolus were counted, provided they did not contact the forbidden lines of the counting frame; motoneuron nucleoli were counted as they appeared while we focused through the z axis, and nucleoli in the first focal plane (i.e., “tops”) were excluded to avoid double counting.

The length of the dissector was approximately 16 μm, which was adequate for visualizing nucleoli in multiple focal planes. Motoneuron counts were derived from a mean of 11.5 sections spaced 480 μm apart and distributed uniformly through the entire rostrocaudal extent of the quadriceps motoneuron pool range. This sampling scheme produced average estimated coefficients of error (CE) of 0.072 for sham animals, 0.061 for SCI animals and 0.066 for SCI+T animals. Cell counts for each animal were corrected for the proportion of sections sampled.

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

Soma volume

To assess potential atrophic changes in motoneurons after SCI, soma volumes were measured. The volume of quadriceps motoneuron somata was assessed in at least one set of alternate sections (160 μm apart) by using the Nucleator method (Gundersen, 1988). A set of four rays emanating from a point randomly chosen within each BHRP-labeled motoneuron soma was drawn and oriented randomly. Soma volumes of an average of 25.8 motoneurons were measured for each animal by using Stereo Investigator at a final magnification of 780×. Average estimated coefficients of error (CEs) were 0.027 for sham animals, 0.023 for SCI animals, and 0.020 for SCI+T animals. Soma volumes within each animal were then averaged for statistical analysis.

Dendritic length

To assess potential atrophic changes in motoneurons after SCI, dendritic lengths and distributions were measured. 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 quadriceps motoneuron dendritic field was assessed in every third section (480 μm apart) in three dimensions by using a computer-based morphometry system (Neurolucida; MBF Bioscience) at a final magnification of 250×. No attempt was made to identify BHRP-labeled fibers as either dendrites or axons. Average dendritic length per labeled motoneuron was estimated by summing the measured dendritic lengths of the series of sections, multiplying by three to correct for sampling, and 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, 1994), after changes in dendritic interactions (Goldstein et al., 1993) and afferent input (Kalb, 1994; Hebbeler et al., 2002; Hebbeler and Sengelaub, 2003), and after injury (Fargo and Sengelaub, 2004a,b, 2007; Little et al., 2009; Wilson et al., 2009).

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 by using a set of axes oriented radially around the center of the collective labeled somata. These axes divided the spinal cord into 12 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 quadriceps motoneuron dendritic arbors. The rostrocaudal extent of the dendritic arbor was determined by recording the rostrocaudal distance spanned by quadriceps motoneuron dendrites for each animal. The maximal radial extent of the arbor in the transverse plane was also measured for each animal, by using the same radial axes and resultant 30° bins used for the dendritic distribution analysis. For each bin, the linear distance between the center of the quadriceps motor pool 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 transverse plane) of BHRP transport to the most distal dendritic processes.

Muscle fiber and motor endplate morphology

To assess potential atrophic changes in muscle after SCI, the target musculature of the quadriceps motoneurons were also examined. The right vastus lateralis muscles were removed immediately after perfusion and weighed. Muscles were then postfixed overnight in the same fixative as used for perfusion, and then transferred to sucrose phosphate buffer (10% w/v, pH 7.4). Muscles were then rinsed in distilled water, blocked into proximal and distal segments, and flash-frozen in 2-methylbutane. Muscle segments were then sectioned (45 μm) either transversely (for examination of muscle fiber cross-sectional area) or longitudinally (for examination of motor endplate area and density) on a cryostat at −20°C and thaw-mounted onto glass slides. Muscle fiber cross-sectional area, a correlate of muscle strength, was assessed after staining with Milligan's trichrome stain. Motor end-plate size and density, measures of muscle innervation, were assessed after staining for acetylcholinesterase by using the Roots–Karnovsky method (Hedreen et al., 1985).

To obtain accurate measures of motor endplate size, only en face profiles were traced. An average of 34.8 muscle fibers and 69.6 endplates were measured for each animal at a final magnification of 510×. The number of motor endplates per muscle fiber was estimated by counting the number of muscle fibers and endplates in a grid (1 × 1 mm), randomly placed on the muscle section (one sample field per section, five muscle sections per animal). An average of 118.00 muscle fibers per animal was examined. Cross-sectional muscle fiber area and motor endplate size and density were measured under brightfield illumination by using Stereo Investigator. Fiber areas and endplate areas and densities within each animal were then averaged for statistical analysis.

Statistical analysis

All data were analyzed by t-tests or analyses of variance (one-way, two-way, or repeated-measures as appropriate) followed by post hoc analyses with Fisher's least significant difference (LSD).

Figure preparation

Digital light micrographs were obtained by using an MDS 290 digital camera system (Eastman Kodak, Rochester, NY). Brightness and contrast of these images were adjusted in Adobe Photoshop (Adobe Systems, San Jose, CA).

RESULTS

Lesion volume and white matter sparing

Contusive SCI resulted in large, centrally located cystic cavities, with thin rims of spared tissue surrounding the cavity (Fig. 1A,B). In both SCI groups, faint staining with Luxol fast blue indicated that contusion injury resulted in areas of demyelination immediately surrounding the lesion cavity. Lesion volumes in blank-implanted animals (6.11 ± 0.69 mm^3; mean ± SEM) did not differ from those of testosterone-treated animals (5.33 ± 0.50 mm^3; $t(9) = -0.89$, ns).

To correct for changes in spinal cord contour that were associated with cavitation after spinal cord contusion, the percent lesion volume was also determined. As for total lesion volume, testosterone treatment had no effect on percent lesion volume (SCI+-blank, 17.29 ± 1.69%; SCI+T, 16.00 ± 1.67%), percent volume of spared white matter (SCI+blank, 62.35 ± 1.19%; SCI+T, 62.80 ± 0.91%), or percent volume of spared gray matter (SCI+blank, 20.36 ± 0.96%; SCI+T, 21.20 ± 0.77%; F(1,18) = 0.02, ns; Fig. 1C). Importantly, despite the large size of the lesions, they did not extend into the L2 level, and thus did not compromise the quadriceps motoneuron populations directly.

Motoneuron counts

In sham animals, the number of motoneurons within the identified quadriceps range averaged 1,054.75 (± 202.96). Contusive SCI with or without testosterone treatment had no effect on the number of quadriceps motoneurons (SCI+blank, 1,077.48 ± 95.58; SCI+T, 1,032.77 ± 36.37; F(2,15) = 0.02, ns).

Motoneuron morphometry

Injection of BHRP into the left vastus lateralis successfully labeled ipsilateral quadriceps motoneurons in all groups (Fig. 2). Labeled motoneurons were located in the lateral motor column in the L2 spinal segment (Nicolopoulos-Stournaras and Iles, 1983; Little et al., 2009). Dendritic arbors were strictly unilateral, with extensive ramification along the ventrolateral edges of the gray matter and in the lateral funiculus, as well as throughout the ventral horn. An average of 47.85 ± 9.13 motoneurons per animal was labeled with BHRP, and did not differ by group (F(2,15) = 2.13, ns).

Soma volume

In sham animals, quadriceps motoneuron somata were typical in size (30,993.61 ± 4,762.39 μm^3), and did not differ from those of SCI+blank (23,667.67 ± 2,835.35 μm^3) or SCI+T animals 29,623.68 ± 1,846.28 μm^3; F(2,15) = 1.12, ns).

Dendritic length

Following contusion injury, quadriceps motoneurons underwent marked dendritic atrophy (Fig. 2). Dendritic length decreased by 41.35% (4,809.03 ± 1,165.46 μm in SCI+blank animals compared with 8,199.59 ± 1,053.87 μm for sham animals, LSD, $P < 0.05$; overall test for the effect of group on arbor per cell F(2,15) = 3.72, $P < 0.05$). However, treatment with testosterone attenuated SCI-induced dendritic atrophy: dendritic lengths in SCI+T animals (9,060.56 ± 1,229.92 μm) were 88.41% longer than those of SCI+blank animals (LSD $P < 0.03$), and did not differ from those of sham animals (LSD, ns).

Dendritic length per bin was non-uniform across radial bins, and a repeated-measures ANOVA revealed a significant effect of radial location (F(11,165) = 10.99, $P < 0.0001$; Fig. 3B). Consistent with the results of the arbor per cell analysis, there was also a significant

effect of group ($F(2,165) = 3.74$, $P < 0.05$). Reductions in dendritic length occurred throughout the radial distribution in SCI+blank animals compared with sham animals (an average of 32.35%, 0° to 300°), and were especially pronounced ventromedially (60.88%, 300° to 360°), resulting in a significant group × location interaction ($F(11,121) = 2.06$, $P < 0.03$). Treatment with testosterone attenuated SCI-induced reductions in dendritic length per bin, and there were no group differences ($F(1,110) = 0.30$, ns) and no group × location interaction ($F(1,110) = 0.40$, ns) between SCI+T animals and sham animals. Dendritic lengths per bin in SCI+T animals were longer than those of SCI+blank animals throughout the radial distribution (an average of 79.65%, 0–300°; $F(1,99) = 6.31$, $P < 0.04$), but again, longer dendritic lengths were found ventromedially (191.76%, 300–360°), resulting in a significant group × location interaction ($F(11,99) = 2.37$, $P < 0.02$).

Dendritic extent

Consistent with the non-uniform dendritic distribution of quadriceps motoneurons apparent in Figure 2, radial dendritic extent differed across bins (Fig. 3C), and repeated-measures ANOVA revealed a significant effect of location ($F(11,165) = 14.35$, $P < 0.0001$). However, radial dendritic extent did not differ across groups ($F(2,165) = 1.00$, ns). Rostrocaudal dendritic extent also did not differ across groups ($F(2,15) = 0.94$, ns), spanning 4,480.00 ± 447.13 μm in sham animals, 5,200.00 ± 397.86 μm in SCI+blank animals, and 4,896.00 ± 139.48 μm in SCI+T animals.

Muscle weight and fiber size

Overall body weight was not affected: animals weighed an average of 259.50 ± 8.8 g at the end of treatment, and this did not differ among groups ($F(2,15) = 3.00$, ns). However, muscle weights were affected by contusion injury; weights of the vastus lateralis muscles decreased by 22.1% (0.84 ± 0.05 g in SCI+blank animals compared with 1.07 ± 0.04 g for sham animals (LSD, $P < 0.002$; overall test for the effect of group on muscle weight $F(2,15) = 7.92$, $P < 0.005$). However, treatment with testosterone attenuated SCI-induced muscle atrophy: weights of the vastus lateralis muscles in SCI+T animals (1.03 ± 0.05 g) were 23.0% larger than those of SCI+-blank animals (LSD $P < 0.02$), and did not differ from those of sham animals (LSD, ns).

Muscle fiber size was also affected by contusion injury. Cross-sectional area of vastus lateralis muscle fibers decreased by 26.3% (1,041.82 ± 141.71 μm^2 in SCI+-blank animals compared with 1,414.46 ± 34.03 μm^2 for sham animals, LSD, $P < 0.05$; overall test for the effect of group on muscle fiber area $F(2,15) = 4.09$, $P < 0.04$). However, treatment with testosterone attenuated SCI-induced muscle fiber atrophy: cross-sectional area of vastus lateralis muscle fibers in SCI+T animals (1,531.53 ± 190.53 μm^2) were 47.0% larger than those of SCI+-blank animals (LSD $P < 0.02$), and did not differ from those of sham animals (LSD, ns).

Motor endplate size and density

Contusion injury with or without hormone treatment had no significant effects on motor endplate size or density. In sham animals, motor endplate areas were typical in size (1,367.30 ± 118.12 μm^2), and did not differ from those of SCI+blank (1,394.98 ± 87.32 μm^2) or SCI+T animals (1,496.88 ± 107.42 μm^2; $F(2,15) = 0.42$, ns). Similarly, the density of motor endplates did not differ across groups (sham animals, 0.68 ± 0.03 endplates per fiber; SCI+blank animals, 0.77 ± 0.03 endplates per fiber, SCI+T animals 0.65 ± 0.05 endplates per fiber; $F(2,15) = 3.25$, ns).

DISCUSSION

Testosterone treatment protects motoneurons from injury-induced atrophy (Fargo et al., 2009a). In this experiment we tested whether testosterone might have similar neuroprotective/neurotherapeutic effects after SCI. Following SCI, surviving motoneurons innervating the quadriceps muscles had significantly decreased dendritic lengths, but this decrease was prevented with testosterone treatment. In addition, testosterone treatment also protected the target muscle weights and fiber areas from SCI-induced decreases. To our knowledge, this is the first time that a pronounced dendritic atrophy in motoneurons has been found in the spinal cord caudal to a contusive injury. More importantly, such atrophy could be almost completely restored after testosterone treatment, indicating a protective role of testosterone on prevention of motoneuron dendritic degeneration after SCI.

Spinal cord lesions

Following contusion, the focal injuries delivered to the T9 spinal cord developed into large lesions that spanned multiple thoracic spinal segments. Four weeks of treatment with testosterone had no effect on lesion volume or tissue sparing. This lack of effect with testosterone treatment is similar to that observed by Kachadroka et al. (2010), wherein the percentage of white matter sparing at the lesion epicenter as a consequence of treatment with estradiol was not affected by the presence or absence of endogenous androgens. However, because the lesions were comparable across SCI groups, the beneficial effects of steroid treatment on the morphology of quadriceps motoneurons and target musculature we observed are not likely a result of differential, long-term tissue sparing.

Lack of effects of gonadal steroid treatment (specifically estradiol) on SCI lesion size at postinjury intervals similar to that used in the current study have been reported previously (3 weeks, Swartz et al., 2007; 4 weeks, Ritz and Hausmann, 2008; but see Chaovipoch et al., 2006). Interestingly, at shorter postinjury intervals, estradiol treatment reduces tissue damage (Srbnick et al., 2005; Ritz and Hausmann, 2008), and this transient early reduction may have allowed for the improved functional outcomes observed at later times (Ritz and Hausmann, 2008). Thus, it is possible that the beneficial effects of testosterone treatment we observed on motoneuron and muscle morphology could similarly reflect an effect of an early, but transient reduction in tissue damage, and examination of shorter postinjury intervals could address this question.

Changes in motoneuron morphology are not direct effects of lesion

Although extensive, spinal lesions did not extend into the lumbar spinal cord, thus sparing the gray matter and resident motoneurons. Counts of either Nissl-stained or BHRP-labeled motoneurons in SCI animals did not differ from those of sham animals, confirming the protection of quadriceps motoneurons from direct damage due to SCI-induced lesions. Similarly, soma size of quadriceps motoneurons was not significantly affected by SCI. Soma size regresses after direct insult to motoneurons, for example, after axotomy (Ma et al., 2002; Yang et al., 2004) or death of neighboring motoneurons (Fargo and Sengelaub, 2004a,b, 2007), and thus, the lack of significant effects on soma size suggests that the quadriceps motoneurons were not directly damaged by SCI-induced lesions. Direct insult to motoneurons, for example through axotomy (Yang et al., 2004) or induced death of neighboring neurons (Little et al., 2009), can result in dendritic atrophy in surviving motoneurons. However, as described above, because the lesion did not infiltrate the L2 level, and the number of Nissl-stained or BHRP-labeled quadriceps motoneurons was not affected by SCI, we do not believe the reductions in dendritic length we observed reflect such direct effects.

Dendritic atrophy after SCI

Afferent input to motoneurons is important for the maintenance of dendritic morphology, and deafferentation often results in dendritic retraction. Following deafferentation via damage to the dorsal horn (Bernstein and Standler, 1983), spinal cord hemisection (Bernstein et al., 1984), or cortical ablation (Standler and Bernstein, 1984), spinal motoneurons undergo dendritic atrophy. Activity in afferent pathways is an important factor in maintaining dendritic morphology. For example, cold block of the spinal cord causes dendritic morphological changes to develop within 4 hours (Castro-Moure and Goshgarian, 1997).

It is possible that the dendritic atrophy we observed following SCI in untreated animals could reflect deafferentation resulting from the loss of descending motor and propriospinal tracts. In rats, the majority of corticospinal tract axons terminate dorsomedially, principally in laminae III–VI (Brown, 1971; Brösamle and Schwab, 1997), whereas those of the rubrospinal tract terminate in laminae V and VI (Waldron and Gwyn, 1969; Brown, 1974). In contrast, reticular formation projections to lumbar levels of the spinal cord, especially from the reticularis pontis oralis, terminate ventromedially, principally in lamina VIII (Motorina, 1977; Jones and Yang, 1985). Propriospinal projections into the lumbar levels also terminate ventromedially, extending into both laminae VII and VIII (Menétey et al., 1985). Following SCI, quadriceps motoneurons in untreated animals showed dendritic atrophy in all of these locations. Interestingly, although reduced dendritic length was present throughout the dendritic distribution, it was particularly pronounced in the ventrome-dial region, where quadriceps motoneuron dendrites normally have a dense ramification into lamina VIII. Because both reticulospinal and propriospinal projections are concentrated in this area, the extensive lesions present after SCI could have produced a major denervation of dendrites in this area, resulting in the pronounced dendritic atrophy we observed. We have previously reported that following spinal transection and the concomitant loss of descending pathways, spinal motoneurons undergo marked local reductions in dendritic arbor, especially in this same region of the ventral horn (Hebbeler and Sengelaub, 2003). This loss is of particular significance after SCI, as descending reticulospinal fibers course through the ventral and lateral funiculi (Jones and Yang, 1985; Martin et al., 1985), and disruption of these tracts results in hindlimb motor deficits (Magnuson et al., 1999; Loy et al., 2002).

Protection of motoneuron dendrites with testosterone

SCI-induced atrophy of quadriceps motoneuron dendrites was attenuated in testosterone-treated animals. Testosterone treatment could have attenuated dendritic atrophy by increasing the number of spared or regenerating axons that traverse the lesion. Such axon sparing has been reported previously with trophic factor treatment (e.g., glia cell line-derived neurotrophic factor; Iannotti et al., 2004), and androgens have been shown to regulate trophic factors such as brain-derived neurotrophic factor (BDNF; Verhovshek et al., 2010) and its high-affinity receptor tyrosine kinase receptor type B (Osborne et al., 2007) in the spinal cord. However, although the sparing of proprio- and supraspinal axons remains possible, the lack of effect on white matter sparing with testosterone treatment we observed does not support this hypothesis.

The attenuation in dendritic atrophy we observed could have been produced by a testosterone-mediated sprouting of quadriceps motoneuron dendrites locally onto remaining afferents. Such sprouting could potentially maintain motor activation, leading to protection of the target musculature from disuse atrophy (see below) and could support exercise training effects on locomotor function after SCI (Raineteau and Schwab, 2001; Gazula et al., 2004). Such an effect of testosterone on attenuating dendritic atrophy and supporting motoneuron activation has in fact been directly demonstrated (Little et al., 2009; Fargo et

al., 2009b). The mechanisms responsible for this sprouting are not clear, but testosterone has been shown to regulate the expression of cytoskeletal proteins (e.g., β-tubulin, Jones and Oblinger, 1994; Matsumoto et al., 1994; Jones et al., 1999b; Brown et al., 2001) as well as neuritin, a critical downstream mediator of the ability of androgens to increase neurite outgrowth (Marron et al., 2005; Fargo et al., 2008a,b). Electrophysiological and anatomical tracing studies could begin to address this question.

Comparability of BHRP labeling

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 treatment groups reflect true dendritic atrophy in quadriceps motoneurons of untreated SCI animals, which is attenuated by treatment with androgens. The possibility that confounds arising from SCI could affect retrograde transport is also an important consideration, as such an artifact could potentially result in apparent alterations in dendritic morphology. However, no differences in either radial or rostrocaudal extents of quadriceps motoneuron dendrites in the SCI groups compared with normal values were observed. Therefore, we believe that the dendritic labeling across groups was comparable and that the shorter dendritic lengths we observed in the untreated SCI animals reflect true dendritic atrophy.

Muscle atrophy after SCI

The regressive changes we observed in muscle weight and fiber diameter are typical after SCI in muscles innervated by motoneurons below the level of the lesion, especially in weight-bearing muscles such as the quadriceps (Peckham et al., 1976; Giangregorio and McCartney, 2006). This atrophy can result from either denervation due to loss of motoneurons or damage to the ventral roots, or disuse consequent to decreases in muscle activation potentially due to the loss of synaptic input to remaining motoneurons (Gordon and Mao, 1994). In the current study, the atrophy we observed cannot be ascribed to an effect of denervation, as we observed no changes in quadriceps motoneuron number, the number of BHRP-labeled quadriceps motoneurons, or the sizes or densities of motor endplates between sham animals and untreated SCI animals. Thus, the decreased weight and fiber size we observed most likely reflect a disuse atrophy, potentially resulting after damage to descending and propriospinal projections and/or the reductions in quadriceps motoneuron dendritic length we observed. Such reductions in quadriceps motoneuron dendritic length result in attenuation of motor activation, reducing response amplitudes in the femoral nerve generated by dorsal root afferent stimulation (Little et al., 2009). Alternatively, disuse atrophy may also result from changes in muscle length or loading conditions that could decrease protein synthesis and increase protein degradation (Goldspink, 1977; Williams and Goldspink, 1973).

Protection of muscle with testosterone

In the current study, testosterone treatment prevented the atrophy in muscle weight and fiber size seen after SCI. Androgens are known to have protein anabolic effects on general skeletal muscle tissue, but these effects are small (Kochakian, 1975). Thus, treatment with testosterone might have supported muscle protein synthesis and decreased protein degradation, and the resultant decrease in protein turnover could have prevented muscle atrophy. However, by using hormone treatments identical to that used in the current study, we have previously demonstrated that the weight of the quadriceps musculature is unaffected in either male (Little et al., 2009; Verhovshek et al., 2010; Huguenard et al., 2011) or female rats (Wilson et al., 2009) by treatment with testosterone.

The protection of muscle weight and fiber size could be the result of a sparing of motoneuron function. As described above, testosterone treatment can reverse the regressive changes in dendritic morphology and motor activation (Little et al., 2009; Fargo et al., 2009b). Thus, the protection from dendritic atrophy with testosterone treatment after SCI could have spared local spinal circuitry sufficiently to maintain motor activation, preventing disuse atrophy of the target muscles. Alternatively, testosterone could have potentially altered mobility or activity in the treated animals, resulting in the preservation of both muscle and the related spinal cord circuitry and motoneuron dendritic morphology. This is quite plausible, as limb exercise after spinal cord transection during postnatal development has in fact been shown to prevent dendritic atrophy in spinal motoneurons (Gazula et al., 2004). Furthermore, exercise is known to elevate the expression of neurotrophic factors (e.g., BDNF) that can promote dendritic and axonal regrowth (Wilhelm et al., 2009). Further studies utilizing behavioral or electrophysiological methods could address this hypothesis directly.

Neuroprotective mechanisms of testosterone

Androgens have been shown to have powerful neuro-protective effects in a variety of systems. For example, testosterone protects against cell death in cultured hippocampal neurons (Pike, 2001), prevents injury-induced dendritic atrophy in cortical pyramidal cells (Forgie and Kolb, 2003), promotes earlier functional recovery after stroke (Pan et al., 2004), and stimulates motoneuron axonal growth after peripheral nerve injury (Kujawa et al., 1989). The mechanisms through which androgens act are multiple, and include attenuation of synaptic stripping (Jones et al., 1997a) and injury-induced upregulation of glial fibrillary acidic protein (GFAP) (Coers et al., 2002; Jones et al., 1997b), mediation of the central glial response (Jones et al., 1999a), and enhancement of the ribosomal response (Kinderman and Jones, 1993). Proteins thought to be involved in neuroprotection are also regulated by androgens, which increase expression of heat shock protein (Zhang et al., 2004; Tetzlaff et al., 2007), proteins with antioxidant functions (e.g., catalase, Ahlbom et al., 2001), and the neurotrophin BDNF (Verhovshek et al., 2010) and its receptor trkB (Osborne et al., 2007). Androgens are also thought to be involved with the activation of neuroprotection signaling pathways (e.g., MAPK/ERK; Pike et al., 2008).

Although testosterone can act directly by activating androgen receptors, it can also act through its conversion to other androgenic or estrogenic steroid hormones. Both androgens and estrogens have protective qualities in the nervous system (Henderson and Reynolds, 2002; Woolley and Cohen, 2002; Bialek et al., 2004). Of direct relevance to the present study, in quadriceps motoneurons, the dendritic atrophy induced by partial motoneuron depletion is attenuated with equal effectiveness by treatment with either testosterone, dihydrotestosterone, or estradiol (Coons et al., 2009). Thus, it is unclear whether the protective effects of testosterone on motoneuron and muscle morphology following SCI were the result of action via androgenic or estrogenic pathways. Establishing which of these mechanisms, proteins, and pathways are involved in the androgen-mediated protection of motoneuron dendrites from SCI-induced atrophy will be valuable contributions to developing new neurotherapeutic strategies.

CONCLUSIONS

Following injury, androgens are already known to reduce motoneuron death or attenuate secondary atrophy and loss of function in surviving motoneurons in several experimental paradigms (Fargo et al., 2009a). The present results indicate that the regressive changes in motoneuron and muscle morphology seen after SCI can be prevented by testosterone treatment, potentially providing a mechanism for the improved locomotor performance previously observed with hormonal treatments following SCI (Yune et al., 2004; Clark et al.,

2008; Ritz and Hausmann, 2008). Together, these results further support a role for testosterone as a neurotherapeutic agent in the injured nervous system.

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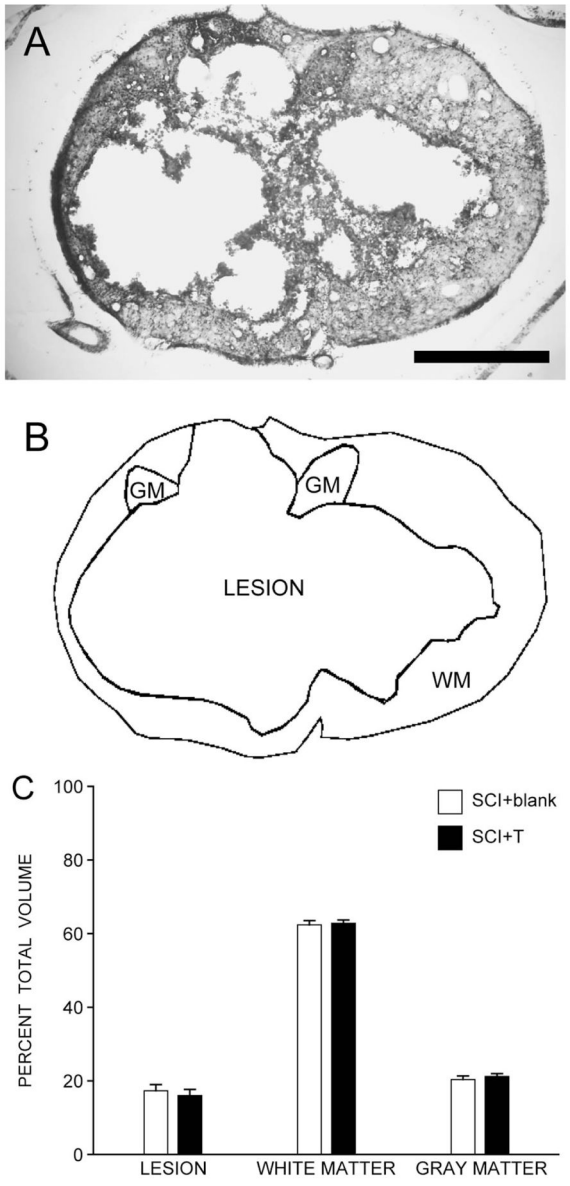

Figure 1.

Histological and stereological analysis of spinal cord spared tissue and lesion volume after contusive SCI with or without testosterone treatment. **A**: Representative section through the lesion epicenter stained with cresyl violet and eosin, showing a large centrally located cystic cavity with a thin rim of spared tissue surrounding the cavity. **B**: Neurolucida drawing from the same section showing the lesion area (including regions of cavitation and fibrosis), residual white matter (WM), and spared gray matter (GM). **C**: Percent total volumes of lesion and spared white and gray matters also did not differ across groups. Bar heights represent means ± SEM. SCI (white bars), n = 6; SCI+T (black bars), n = 5. Scale bar = 500 μm in A.

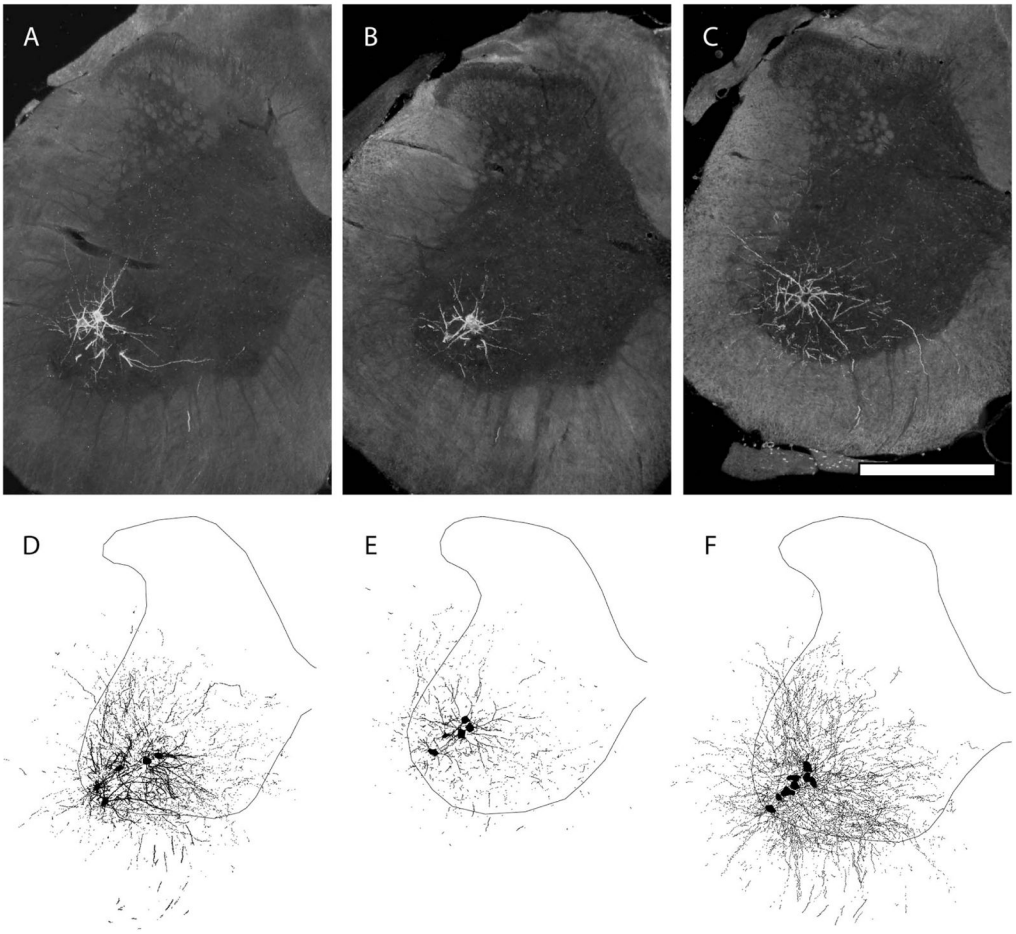

Figure 2.

Darkfield digital micrographs and matching computer-generated composites of transverse hemisections through the lumbar spinal cords of a sham animal (**A,D**), an injured animal given a blank implant (SCI+blank, **B,E**), and a testosterone-treated injured female (SCI+T, **C,F**), after BHRP injection into the left vastus lateralis muscle. Computer-generated composites of BHRP-labeled somata and processes were drawn at 480-μm intervals through the entire rostrocaudal extent of the quadriceps motor pool; these composites were selected because they are representative of their respective group average dendritic lengths. Scale bar = 500 μm in C (applies to A–C).

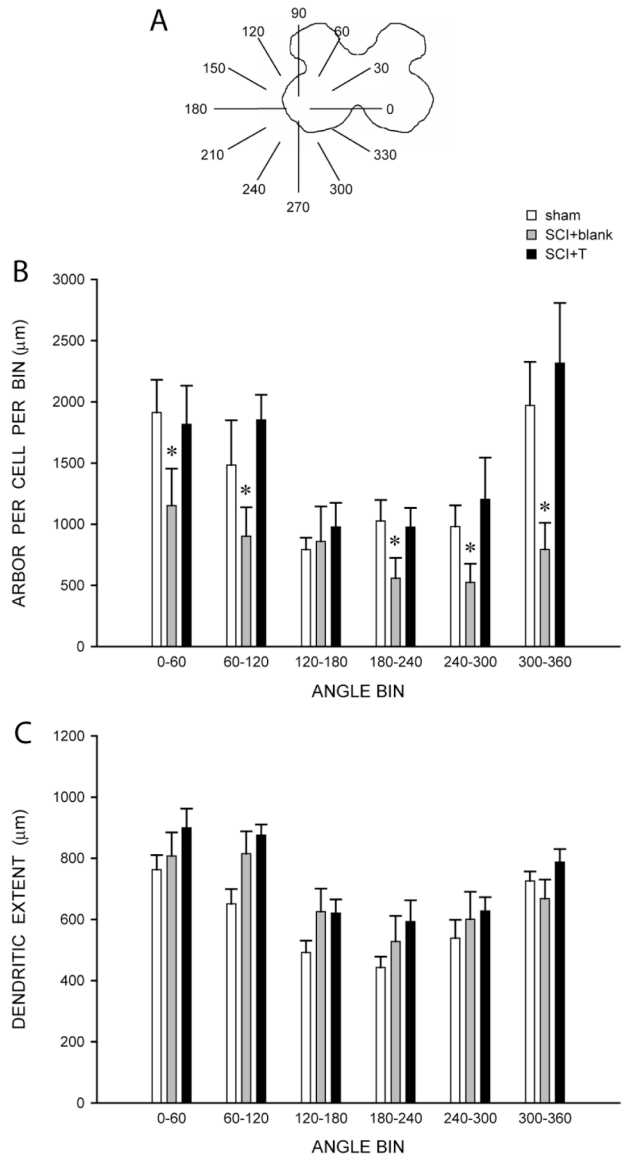

Figure 3.

A: Drawing of spinal gray matter divided into radial sectors for measure of quadriceps motoneuron dendritic distribution and maximal radial extent. **B**: Quadriceps motoneuron dendritic arbors display a non-uniform distribution, with the majority of the arbor located between 300° and 120°. Following contusion injury, surviving quadriceps motoneurons in untreated animals (SCI) had reduced dendritic lengths throughout the radial distribution, especially ventromedially (60.88%, 300°–360°). Treatment with testosterone (SCI+T) attenuated these reductions. **C**: Following contusion injury, extent measures of surviving quadriceps motoneurons in SCI+blank and SCI+T animals did not differ from those of sham animals, demonstrating a comparable degree of dendritic labeling. For graphic purposes, measures of dendritic length and extent have been collapsed into six bins of 60° each. Bar heights represent means ± SEM. Sham animals (white bars), n = 7; SCI (gray bars), n = 6; SCI+T (black bars), n = 5.