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Neuroprotective effects on the morphology of somatic motoneurons following the death of neighboring motoneurons: A role for microglia?

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Abstract

Partial depletion of spinal motoneuron populations induces dendritic atrophy in neighboring motoneurons, and treatment with testosterone protects motoneurons from induced dendritic atrophy. We explored a potential mechanism for this induced atrophy and protection by testosterone, examining the microglial response to partial depletion of motoneurons. Motoneurons innervating the vastus medialis muscles of adult male rats were killed by intramuscular injection of cholera toxin-conjugated saporin; some saporin-injected rats were treated with testosterone. Microglia were later visualized via immunohistochemical staining, classified as monitoring or activated, and counted stereologically. Partial motoneuron depletion increased the number of activated microglia in the quadriceps motor pool, and this increase was attenuated with testosterone treatment.

The attenuation in microglial response could reflect an effect of testosterone on suppressing microglia activation, potentially sparing motoneuron dendrites. Alternatively, testosterone could be neuroprotective, sparing motoneuron dendrites, secondarily resulting in reduced microglial activation. To discriminate between these hypotheses, following partial motoneuron depletion, rats were treated with minocycline to inhibit microglial activation. Motoneurons innervating the ipsilateral vastus lateralis muscle were later labeled with cholera toxin-conjugated horseradish peroxidase, and dendritic arbors were reconstructed. Reduction of microglial activation by minocycline did not prevent induced dendritic atrophy following partial motoneuron depletion. Further, reduction of microglial activation by minocycline treatment resulted in dendritic atrophy in intact animals. Together, these findings indicate that the neuroprotective effect of testosterone on dendrites following motoneuron death is not due inhibiting microglial activation, and that microglial activity contribute to the normal maintenance of dendritic arbors.

Keywords

steroids; neuroprotection; morphology; dendrites

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INTRODUCTION

Neurodegenerative disease or injury often results in the loss of spinal motoneurons. For example, motor neuron diseases (e.g., amyotrophic lateral sclerosis; Cleveland and Rothstein, 2001) and spinal muscular atrophies (e.g., spinal and bulbar muscular atrophy; Kennedy et al., 1968) are characterized by progressive loss of motoneurons. The loss of spinal motoneurons, results in muscle weakness, loss of muscular control, and can eventually cause complete paralysis (Cleveland and Rothstein, 2001). Alternatively, spinal cord injury (Liu et al., 1997) or damage to spinal nerves resulting in laceration and avulsion of spinal roots (e.g., cauda equina injury; 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).

Importantly, after such insults surviving motoneurons also show a variety of morphological and functional changes. For example, motoneurons undergo dendritic retraction and atrophy after injury (e.g., after spinal cord injury; Byers et al., 2012; Liu, 2014a,b). Similarly, after peripheral axotomy motoneurons show functional and biochemical changes (e.g., Titmus and Faber, 1990; Bisby and Tetzlaff, 1992) as well as dendritic atrophy (e.g., Sumner and Watson, 1971; O'Hanlon and Lowrie, 1995; Yang et al., 2004).

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 dendritic atrophy (Fargo and Sengelaub, 2004a,b, 2007; Little et al., 2009; Wilson et al., 2009; Cai et al., 2017). This secondary atrophy is responsible for at least some of the movement deficits that accompany degenerative movement disorders and spinal cord trauma, as it results in reduced excitability of the remaining motoneurons (Fargo et al., 2009b; Little et al., 2009). 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.

Motoneurons display remarkable plasticity in adulthood and are capable of remodeling their cellular morphology; this propensity for change may allow for the development of treatment regimens in neural disease and injuries (Edgerton et al., 2004). Treatment with gonadal steroid hormones promotes a wide array of neuroprotective and neurotherapeutic effects (Jones et al., 2001; Fargo et al., 2009a; Foecking et al., 2015). 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 the rate of axon regeneration (Kujawa et al., 1991) as well as the recovery of motor function (Kujawa and Jones, 1990; Kujawa et al., 1989). Using our model of motoneuron loss, we have begun to examine the neuroprotective effects of gonadal steroids on the secondary atrophy induced in neighboring surviving motoneurons. Our previous experiments have demonstrated that testosterone is indeed neuroprotective following motoneuron loss. Treatment with exogenous testosterone attenuates dendritic atrophy, as well as the attenuated excitability, in motoneurons induced by motoneuron loss in both highly androgen-sensitive (Fargo and

Sengelaub, 2004a,b, 2007; Fargo et al., 2009b) as well as more typical somatic (Little et al., 2009; Wilson et al., 2009; Cai et al., 2017) motoneuron populations.

In the present study, we explored a potential mechanism for this induced dendritic atrophy and protection by androgen treatment, examining the response of microglia to the partial depletion of motoneurons. In their basal state, microglia monitor the health of dendritic spines and synapses (Trapp et al., 2007; Wake et al., 2009; Tremblay et al., 2011; Salter and Beggs, 2014). Microglia also function as the resident immune cells of the central nervous system. After injury, microglia shift from the “monitoring” state to an active immunocompetent, phagocytic state; these activated microglia then migrate to the site of insult (Loane and Byrnes, 2010; Kettenmann et al., 2013). Microglia are influenced by a series of ‘on’ and ‘off’ signals that can induce shifts between their active and monitoring states (Biber et al., 2007). ‘On’ signals induce shifts to the active morphology and function, and such signals include a variety of inflammatory cytokines (IL-6, IL-1 β , TNF- α), purines (ATP), glutamate, and matrix metalloproteinases (MMPs) that are released from apoptotic cells; ‘off’ signals include neuron-microglia conjugate-signaling using CX3CL1-CX3CR1, non-glutamatergic neurotransmitter signaling, and certain anti-inflammatory cytokines (IL-10; TGF- β ; Biber et al., 2007; Loane and Byrnes, 2010). In their immunocompetent, phagocytic state, microglia contribute to the inflammatory immune response, release damaging reactive oxygen species, and phagocytose cellular debris (Gehrmann et al., 1993; Loane and Byrnes, 2010; Neher et al., 2012; Kettenmann et al., 2013). Moreover, microglial recruitment via chemokine CXCR3 signaling is necessary for post-injury loss of dendritic branches (Rappert et al., 2004), further suggesting a role for activated microglia in producing the secondary atrophy induced in neighboring surviving motoneurons.

Microglia have also been shown to be sensitive to testosterone. Following a stab wound brain injury, the number of microglia at the injury site is elevated, and treatment with testosterone significantly reduces the number of microglia (Barreto et al., 2007). Thus, the attenuation in dendritic atrophy with testosterone treatment we have reported previously (Fargo and Sengelaub, 2004a,b, 2007; Fargo et al., 2009b; Little et al., 2009; Wilson et al., 2009; Cai et al., 2017) could reflect a similar testosterone-mediated reduction in microglial response. However, microglia have also been shown to contribute to tissue repair and provide trophic support to the damaged cellular environment (Streit, 2002; Neumann et al., 2008; David and Kroner, 2011). Thus, it remains uncertain whether activated microglia are ultimately harmful or beneficial to recovery following injury.

It is possible that following induced death of motoneurons, activated microglia respond to remove cellular debris but also cause dendritic atrophy in surviving motoneurons. The neuroprotective effects of testosterone in attenuating dendritic atrophy could be attributable to an effect of testosterone directly suppressing microglia activation, potentially sparing motoneurons from dendritic loss. Alternatively, testosterone could have a more neuroprotective effect, directly sparing the dendrites of surviving motoneurons from induced dendritic atrophy, secondarily resulting in reduced microglial activation. In the present study, we conducted two experiments to investigate the mechanism of testosterone’s neuroprotective effects. We first examined whether there was an upregulation of activated microglia following partial motoneuron depletion, and whether that upregulation could be

moderated by testosterone treatment. We then examined whether inhibition of microglial activation after partial motoneuron depletion protected motoneurons from induced dendritic atrophy, similar to that seen with testosterone treatment.

METHODS

Experiment 1: Does testosterone treatment attenuate microglial activation following partial motoneuron depletion?

In rats, the quadriceps muscles of the thigh comprises the vastus lateralis, vastus medialis, vastus intermedialis, and the rectus femoris (Hebel and Stromberg, 1986). These muscles are 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) and project via the femoral nerve to the four muscles of the quadriceps. The distribution of somata and dendritic arbors of the motoneurons innervating the individual muscles of the ipsilateral quadriceps complex overlap extensively in the lateral motor column (Nicolopoulos-Stournaras and Iles, 1983; Sengelaub et al., 2006), making it possible to partially and selectively deplete this motor population and study the effects of that depletion on the surviving motoneurons.

To examine the response of microglia to partial motoneuron depletion, and the potential effects of testosterone treatment on that response, motoneurons were selectively killed by intramuscular injection of the toxin saporin. Microglia were later visualized via immunohistochemical staining and counted stereologically.

Animals—Adult male Sprague-Dawley rats (approximately 100 days old; Envigo, Indianapolis) were maintained on a 12:12-hour light/dark cycle with unlimited access to food and water. We used the toxin saporin, conjugated to the cholera toxin B subunit, to kill motoneurons selectively. Saporin is a ribosome-inactivating protein; it kills cells by irreversibly inactivating ribosomes thereby halting protein synthesis (Stirpe et al., 1983, 1992). Cholera toxin-conjugated saporin is retrogradely transported from the site of injection and kills motoneurons innervating the injected musculature within 3–6 days (Fargo and Sengelaub, 2004a).

Rats were anesthetized with isoflurane, and motoneurons innervating the left vastus medialis muscle were selectively killed by intramuscular injection of cholera toxin-conjugated saporin (2 μ l, 0.1%; Advanced Targeting Systems, Inc., San Diego, CA). Some of the rats were not treated further (n=12) whereas others were implanted with subcutaneous Silastic capsules with testosterone (4-androsten-17 β -ol-3-one; Steraloids; 3.18mm O.D., 1.57 I.D., 45 mm long) immediately following saporin injection (n=12). Such implants produce testosterone plasma titers in the 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, 2007; Little et al., 2009; Wilson et al., 2009). Testosterone implants were left in place until animals were euthanized.

Half of the animals in each of the saporin-injected groups were allowed to survive for either one or three weeks following saporin. These time points were chosen based on our previous

work wherein we found that atrophy in surviving quadriceps motoneuron dendrites occurs linearly after induced motoneuron death, and is maximal at four weeks after saporin injection (Coons et al., 2009). An additional group of untreated animals was also included (n=6).

Iba-1 immunohistochemistry—The motoneurons innervating the quadriceps muscles do not form a discrete nucleus, but instead are contained within a large continuous population of motoneurons in the lateral motor column. Thus, to identify the appropriate location in the lateral motor column for subsequent analyses, we used a method similar to that of Osborne et al., (2007). The location of quadriceps motoneurons was identified by retrograde labeling using horseradish peroxidase conjugated to the cholera toxin B subunit (BHRP; List Biological Laboratories, Campbell, CA). Two days prior to sacrifice, animals were anesthetized with isoflurane, and the right (i.e., contralateral to the saporin-injected muscle) vastus lateralis muscle was exposed and injected with BHRP (2 μ L, 0.2%). Forty eight hours after BHRP injection, a period that ensures optimal labeling of motoneurons (Kurz et al., 1986; Goldstein et al., 1990), animals were weighed, given an overdose of urethane (approximately 0.25 g/100 g body weight), and perfused intracardially with 0.1M phosphate buffered saline (PBS) followed by cold fixative (4% paraformaldehyde). To confirm the efficacy of saporin injections, the vastus medialis muscles were removed bilaterally immediately after perfusion and weighed. The lumbar portion of the spinal cord of each animal was removed, postfixed in the same fixative for 18 hours, and then transferred to sucrose phosphate buffer (30% w/v, pH 7.4) overnight for cryoprotection. Spinal cords were then frozen and sectioned horizontally at 40 μ m; all sections were collected into three alternate series. BHRP labeling was visualized immediately in one series of sections using a modified tetramethyl benzidine protocol (Mesulam, 1982), mounted on gelatin-coated slides, and counterstained with neutral red. These sections were then examined under light microscopy for the presence of BHRP-labeled quadriceps motoneurons and the appropriate matching sections in the remaining two alternate series were selected for immunohistochemical processing.

Microglia were visualized in the remaining alternate sections using a protocol adapted from Lenz et al. (2013) and Bollinger et al. (2016). Free-floating sections were rinsed in 0.1M PBS, blocked 1 hour in 10% bovine serum albumin (BSA) in PBS + 0.4% Triton X (PBST), and incubated in 0.3% hydrogen peroxide in 50% methanol 1 hour at 4°C. Sections were then rinsed and incubated overnight at 4°C with an antibody specific to ionized calcium-binding adaptor protein-1 (Iba-1, 1:5000; Wako Chemicals Inc., Richmond, VA) in 5% BSA + PBST. Iba-1 is constitutively expressed in microglia, involved in cytoskeletal reorganization, and up-regulated in response to microglial cell activation (Imai and Kohsaka, 2002). Sections were then rinsed, incubated 1 hour at room temperature with biotinylated secondary antibody (1:500, Vector Laboratories Inc., Burlingame, CA) in 5% BSA + PBST, rinsed, and incubated 1 hour with ABC complex (Vector Laboratories Inc.) in PBST. Iba-1-immunopositive cells were visualized using a nickel-intensified DAB reaction. Sections were subsequently rinsed, mounted, counterstained with neutral red, and coverslipped.

Motoneuron and Microglial Counts—In order to determine the rostrocaudal limits of the area of interest within the lateral motor column, the locations of BHRP-labeled motoneurons were plotted using Stereo Investigator (MBF Bioscience, Williston, VT). The medial limit of the area of interest was set at 480 μm from the lateral margins of the gray matter, based on previous methods from our laboratory for counting quadriceps motoneurons (Little et al., 2009; Byers et al., 2012). Estimates of the total number of motoneurons in the left and right lateral motor columns were obtained from the series of sections collected for BHRP labeling using the optical fractionator method as described previously (Little et al., 2009; Byers et al., 2012). Counts were made bilaterally at $\times 937.5$ under brightfield illumination. Quadriceps motoneurons are easily recognizable as large, darkly staining, multipolar cells, and were counted regardless of BHRP labeling (if present). A counting frame ($100 \mu\text{m} \times 100 \mu\text{m}$) was moved systemically throughout the areas of interest in the lateral motor columns in each section (approximately $750,000 \mu\text{m}^2$) of the ventral horn (sections spaced $120 \mu\text{m}$ apart); motoneurons were counted, provided they did not contact the forbidden lines of the counting frame. Motoneurons were counted as they appeared while focusing through the z axis, and cells in the first focal plane (i.e., “tops”) were excluded to avoid double counting. The length of the disector was approximately $20 \mu\text{m}$. This sampling scheme produced average estimated coefficients of error (CE) of .09. Cell counts for each animal were then expressed as a ratio (motoneuron number on the saporin-injected side relative to that on the untreated side) to quantify the magnitude of motoneuron depletion.

The area of interest for microglial counts was determined using the location of the contralaterally BHRP-labeled quadriceps motoneurons. For each animal, the rostrocaudal limits of the quadriceps motoneuron distribution were superimposed contralaterally over the corresponding immunohistochemically-stained sections (Fig. 1). Microglia were counted and classified as having either “monitoring” (a.k.a, ramified: small, rounded soma, thin, radially branching processes), or “activated” [including primed (polarized soma, thicker processes with less branching), reactive (oblong soma with thick processes with no branching), or amoeboid (rounded soma with no branching) microglial cells] phenotypes based on standardized morphological criteria at a final magnification of $\times 937.5$ (Ransohoff and Perry, 2009; Bollinger et al., 2016). Estimates for the number of microglia were obtained using Stereo Investigator’s optical fractionator probe and a counting frame of $100 \mu\text{m} \times 100 \mu\text{m}$, with guard zones set using a probe-center thickness of $8 \mu\text{m}$. This sampling scheme produced average estimated coefficients of error (CE) of .08. The total number and the ratio of activated to monitoring microglia was determined for each animal.

Experiment 2: Does minocycline administration attenuate secondary dendritic atrophy following partial motoneuron depletion

To examine the potential role of activated microglia in producing the dendritic atrophy we have observed following partial motoneuron depletion, motoneurons were selectively killed by intramuscular injection of the toxin saporin. Microglial activation was then suppressed by treatment with minocycline, and dendritic morphology of quadriceps motoneurons was assessed.

Animals—Adult male Sprague-Dawley rats (approximately 100 days old; Envigo, Indianapolis) were maintained on a 12:12-hour light/dark cycle with unlimited access to food and water. To determine whether inhibition of microglial activation could protect motoneurons from dendritic atrophy following partial motoneuron depletion, motoneurons innervating the left vastus medialis muscle were selectively killed using the saporin protocol described in Expt. 1 and subsequently treated with minocycline (n=6; see below). Minocycline was administered for 4 weeks, a length of time sufficient to observe saporin-induced effects on motoneuron morphology (Fargo and Sengelaub, 2004a, b; 2007; Little et al., 2009; Cai et al., 2017). To control for potential effects of minocycline treatment alone, a group of intact (no saporin injection) animals was treated with minocycline (n=5). To allow direct comparisons to the effects of saporin treatment alone (n=6), that of saporin and subsequent testosterone treatment (n=6), or no treatment (untreated animals, n=5), data collected previously (Little et al., 2009) was included (overall n=28, 5–6 animals per group as detailed above).

Minocycline Administration—Minocycline hydrochloride (PCCA, Houston) was administered orally via the rats' drinking water at a dosage of 40 mg/kg of body weight/day (Hinwood et al., 2012a,b). Consumption of drinking water was monitored prior to any manipulation in order to determine the necessary concentration of minocycline solution in order to meet daily dosage requirements. Minocycline solution (480 mg of minocycline hydrochloride dissolved in 1 L of drinking water) was available ad libitum following saporin injection and until sacrifice. Minocycline solution consumption was monitored daily over the four week treatment period in order to confirm that animals received their intended daily dosage of minocycline; daily minocycline solution consumption did not significantly differ from normal water consumption, indicating that the animals received proper minocycline dosages and that minocycline did not affect voluntary fluid consumption.

Use of minocycline has been previously validated as an inhibitor of monocyte and microglial function (Tikka et al., 2001; Hinwood et al., 2012a,b); microglia are descendent of early myeloid progenitor cells and are the resident monocytes of the central nervous system (Gordon and Taylor, 2005; Ginhoux et al., 2010). Although minocycline has been criticized because it non-specifically inhibits microglial function, our study did not aim to selectively target microglia; rather, our goal was to determine whether microglial activation is necessary rather than sufficient to cause secondary dendritic atrophy. Thus, non-specific inhibition of general monocyte activity is not a primary concern in our methodology.

In order to confirm that the minocycline treatment regimen successfully dampened microglial activation, we first assessed microglial activation in a separate group of animals. Motoneurons innervating the left vastus medialis muscle were selectively killed using the saporin injection protocol described in Expt. 1. One group of saporin-injected animals received no further treatment (n=8), and another was treated with minocycline as described above (n=5). An additional group of untreated animals was also included (n=5). The location of quadriceps motoneurons was identified by retrograde labeling with BHRP as described in Expt. 1. One week after saporin injection, microglia were visualized immunohistochemically as described in Expt. 1 and their density was assessed. Briefly, the area of interest for microglial counts was determined using the location of the BHRP-labeled quadriceps

motoneurons. For each section (8 sections per animal), a counting frame ($270\ \mu\text{m} \times 270\ \mu\text{m}$) was arbitrarily placed in the center of the area of interest, and microglia were counted and classified by phenotype as described in Expt. 1 at a final magnification of $\times 937.5$. The densities for total microglia and the ratio of activated to monitoring microglia was then determined for each animal.

Histochemical and Histological Processing—Four weeks after saporin injection, animals were reanesthetized, and the left vastus lateralis muscle (ipsilateral to the saporin-injected vastus medialis in saporin animals) was injected with BHRP using the protocol described in Expt. 1. BHRP labeling permits population-level quantitative analysis of motoneuron somal and dendritic morphologies (Kurz et al, 1986; Goldstein et al, 1990). Forty-eight hours after BHRP injection, animals were weighed, given an overdose of urethane ($0.25\ \text{g}/100\ \text{g}$ body weight), and perfused intracardially with saline followed by cold fixative (1% paraformaldehyde/1.25% glutaraldehyde). To confirm the efficacy and specificity of saporin injections, the vastus lateralis and vastus medialis muscles were removed bilaterally immediately after perfusion and weighed. The lumbar portion of the spinal cord of each animal was removed, postfixed in the same fixative for 5 hours, 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 $40\ \mu\text{m}$; 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 with tetramethyl benzidine, mounted on gelatin-coated slides, and counterstained with thionin.

Motoneuron Number and Morphology

Motoneuron Counts. 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. Motoneurons were then counted 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 using the optical disector method as previously described (Little et al., 2009). Counts were made at $\times 937.5$ under brightfield illumination. Quadriceps motoneurons are easily recognizable as large, darkly staining, multipolar cells.

A counting frame ($110\ \mu\text{m} \times 80\ \mu\text{m}$) was moved systematically throughout an area of each ventral horn ($\sim 500\ \mu\text{m} \times 500\ \mu\text{m}$, defined by the actual distribution of BHRP-labeled somata) 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 focusing through the z-axis, and nucleoli in the first focal plane (i.e., “tops”) were excluded to avoid double counting. The length of the disector was approximately $16\ \mu\text{m}$, which was adequate

for visualizing nucleoli in multiple focal planes. Motoneuron counts were derived from a mean of 13.0 sections spaced 160 μm apart and distributed uniformly through the entire rostrocaudal extent of the quadriceps motoneuron pool range. This sampling scheme produced an average estimated coefficient of error (CE) of .04. Cell counts for each animal were corrected for the proportion of sections sampled, and then expressed as a ratio (motoneuron number on the saporin-injected side relative to that on the untreated side) to quantify the magnitude of motoneuron depletion.

Using similar methods, the number of BHRP-filled 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, where somata could be visualized and cytoplasmic inclusion of BHRP reaction product confirmed.

Dendritic Length.: For each animal, dendritic lengths in a single representative set of alternate series 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 arbor was assessed; BHRP labeling spanned a rostrocaudal distance of an average of over 3750 μm . BHRP-labeled fibers were traced in every third section (480 μm apart) in three dimensions using a computer-based morphometry system (Neurolucida, MBF Bioscience) at a final magnification of $\times 250$. 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, 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 it 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 neighboring motoneurons (Fargo and Sengelaub, 2004a,b; Little et al., 2009; Cai et al., 2017) or spinal cord injury (Byers et al., 2012; Sengelaub et al., 2018).

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 center of the collective labeled somata. 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), afferent input (Hebbeler et al., 2002; Hebbeler and Sengelaub, 2003), or spinal cord injury (Byers et al., 2012; Sengelaub et al., 2018).

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 labeled dendrites for each animal. The maximal radial extent of the arbor in the transverse 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 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.

All procedures were performed in accordance with the Indiana University Animal Care and Use Guidelines. All data were analyzed by t-tests or analyses of variance followed by post hoc analyses using Fisher's least significant difference (LSD). Digital light micrographs were obtained using an MDS 290 digital camera system (Eastman Kodak Company, Rochester, NY). Brightness and contrast of these images were adjusted in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

RESULTS

Experiment 1

Muscle Weights—Differences in body weight were present across groups [$F(4,25) = 4.33$, $p < .009$], and thus raw muscle weights were corrected for body mass to assess potential effects of saporin and/or hormone treatment effects on muscle weight (Fig. 2). In untreated animals, the corrected weights of the right ($0.160 \pm .007$, mean $\pm$ SEM) and left ($0.165 \pm .008$) vastus medialis muscles were similar [$t(5) = 1.46$, *ns*]. Although the weights of the uninjected (right) vastus medialis muscles were not affected [$F(4,25) = 2.38$, *ns*], injection of saporin into the left vastus medialis resulted in marked muscle atrophy across the saporin groups [overall average of 53% reduction in weight; $F(4,25) = 21.82$, $p < .0001$]. Compared to those of untreated animals, saporin-injected animals had vastus medialis weights that were 59% and 53% (one and three weeks respectively) lighter (LSDs, $ps < .0001$). Treatment with testosterone did not prevent muscle weight loss; compared to those of untreated animals, testosterone-treated saporin animals had vastus medialis weights that were 52% and 50% (one and three weeks respectively) lighter (LSDs, $ps < .0001$). Muscles weights across all saporin groups did not differ from each other [$F(3,20) = 0.69$, *ns*].

Motoneuron Counts—In untreated animals, the number of motoneurons within the identified quadriceps range did not differ between the left and right motor columns [paired t-test, $t(5) = -.575$, *ns*]. Motoneuron counts indicated that saporin was effective in inducing partial motoneuron depletion from the quadriceps motor pool (Fig. 3). Injection of saporin into the left vastus medialis muscle resulted in the death of ipsilateral quadriceps motoneurons, significantly reducing the number of motoneurons in the left motor column relative to that in the right [$F(4,25) = 3.70$, $p < .017$]. Unilateral injection of saporin into the left vastus medialis resulted in an average of 25% reduction in the relative number of motoneurons at one and three weeks compared with that of untreated animals (LSDs, $ps < .$

038). Treatment with testosterone did not prevent this reduction at either time point (overall average of 20% reduced, LSDs, $p < .033$ compared to untreated animals); the reduced relative number of motoneurons within the quadriceps range did not differ across saporin groups [$F(3,20) = 2.32$, *ns*].

Microglial Counts—Microglial cells were successfully labeled with Iba-1, and as described above, classified as having either monitoring or activated (including primed, reactive, or amoeboid) phenotypes based on standardized morphological criteria (Ransohoff and Perry, 2009; Bollinger et al., 2016; see Fig. 4). At one week after saporin injection, the total number of microglia in the quadriceps area of interest was significantly increased (110%) in saporin-injected animals (344.93 ± 40.12) compared to that of untreated animals (164.29 ± 9.54 ; LSD, $p < .0006$; Fig. 5A). Treatment with testosterone reduced saporin-generated microgliosis; the total number of microglia (288.65 ± 29.74) was elevated only 76% above untreated levels (LSD, $p < .009$), but the total number of microglia did not differ across saporin groups [LSD, *ns*; overall ANOVA $F(2,15) = 9.92$, $p < .002$]. At three weeks after saporin injection, the total number of microglia in the saporin-injected animals did not differ from those of untreated animals, and testosterone treatment had no effect (LSDs, *ns*) or across saporin groups [LSD, *ns*; overall ANOVA $F(2,15) = 1.01$, *ns*].

Changes in the number of microglia were further explored by expressing the number of activated phenotypes relative to the monitoring form (Torres-Platas et al., 2014; Bollinger et al., 2016; Fig. 5B). Similar to the results described above, at one week after saporin injection, the relative number of activated microglia in the quadriceps area of interest was significantly increased (1020%) in saporin-injected animals (27.50 ± 6.50) compared to that of untreated animals ($2.46 \pm .38$; LSD, $p < .0005$). Treatment with testosterone reduced saporin-generated microgliosis at one week after saporin injection; the relative number of activated microglia (11.19 ± 2.19) was elevated only 356% over levels found in untreated animals (LSD, *ns*). Further, the relative number of activated microglia in testosterone-treated saporin animals was significantly less (59% fewer) than that of saporin-injected animals [LSD, $p < .011$; overall ANOVA $F(2,15) = 10.29$, $p < .002$]. At three weeks after saporin injection, the relative number of activated microglia in the saporin-injected animals did not differ from those of untreated animals, and testosterone treatment had no effect (LSDs, *ns*) or across saporin groups [LSD, *ns*; overall ANOVA $F(2,15) = 1.99$, *ns*].

Experiment 2

Confirmation of Minocycline Treatment Efficacy—Minocycline treatment was successful in attenuating microglial recruitment after induced motoneuron death (Fig. 6). Analysis revealed a significant effect of treatment group [$F(2,15) = 9.38$, $p < 0.003$] on the density of total microglia (Fig. 7A). Saporin-injected animals displayed a significantly greater density (54.07 ± 9.19 per $100 \mu\text{m}^3$) of total microglia compared to that of untreated animals (22.33 ± 4.78 per $100 \mu\text{m}^3$, an increase of 142%; LSD, $p < .01$), indicating that saporin-induced motoneuron death resulted in recruiting additional microglia. This elevation in total microglia was prevented in minocycline-treated saporin animals; the density of total microglia (11.10 ± 1.03 per $100 \mu\text{m}^3$) did not differ from that of untreated animals (LSD,

ns). Further, the density of total microglia in minocycline-treated saporin animals was significantly lower (79%) than that of saporin-injected animals [LSD, $p < .002$;

As performed in Expt. 1, changes in the density of microglia were further explored by expressing the number of activated phenotypes relative to the monitoring form (Fig. 7B). As found for the density of total microglia, a significant effect of treatment was observed in the density of the relative number of activated microglia [$F(2,15) = 5.54$, $p < .02$]. Saporin-injected animals had a significantly greater relative density of activated microglia in the quadriceps area of interest ($.22 \pm .04$ per 100 μm^3 , increased by 112%) compared to untreated animals ($.10 \pm 0.03$ per 100 μm^3 ; LSD, $p < .02$). This elevation in the relative density of activated microglia was prevented in minocycline-treated saporin animals, and the relative density of activated microglia ($.09 \pm .01$ per 100 μm^3) did not differ from that of untreated animals (LSD, *ns*). Further, the relative density of activated microglia in minocycline-treated saporin animals was significantly lower (58%) than that of saporin-injected animals (LSD, $p < .02$), again confirming that the minocycline treatment regimen successfully dampened microglial activation.

Muscle Weights—Raw muscle weights were corrected for body mass to assess potential effects of saporin, hormone, and minocycline treatments on muscle weight (Fig. 8). In untreated animals, the corrected weights of the right ($0.17 \pm .01$) and left ($0.17 \pm .01$) vastus medialis muscles were similar [paired t-test, $t(4) = 2.14$, *ns*]. Although the weights of the uninjected (right) vastus medialis muscles were not affected [$F(4,24) = 1.09$, *ns*], unilateral injection of saporin into the left vastus medialis resulted in marked atrophy of the injected muscle across saporin groups [average 71% reduction in weight; $F(4,24) = 126.48$, $p < .0001$]. Compared to those of untreated animals, saporin-injected animals had vastus medialis weights that were 74% lighter (LSD, $p < .0001$). Treatment with testosterone or minocycline did not prevent muscle weight loss across all saporin-treated groups (LSDs, $p\text{s} < .0001$ compared to untreated animals) and muscle weights did not differ across saporin-treated groups [$F(2,15) = 1.19$, *ns*].

Notably, the effect of saporin injection on quadriceps weight was specific to the injected muscle. In untreated animals, the corrected weights of the right ($0.40 \pm .02$) and left ($0.41 \pm .02$) vastus lateralis muscles were similar [paired t-test, $t(4) = .43$, *ns*]. The weights of the vastus lateralis muscles on the untreated (right) side did not differ across groups [$F(4,23) = .19$, *ns*]. Most importantly, the weights of the vastus lateralis muscles adjacent to the saporin-injected vastus medialis muscles also did not differ across groups [$F(4,23) = .36$, *ns*]. Treatment with testosterone or minocycline had no effect on vastus lateralis muscle weight (LSDs, *ns*).

Motoneuron Counts—In untreated animals, the number of motoneurons within the identified quadriceps range did not differ between the left (251.2 ± 14.28) and right (237.6 ± 24.97) motor column [paired t-test, $t(4) = 0.63$, *ns*]. Motoneuron counts indicated that saporin was effective in inducing partial motoneuron depletion from the quadriceps motor pool (Fig. 9). Injection of saporin into the left vastus medialis muscle resulted in the death of ipsilateral quadriceps motoneurons, significantly reducing the number of motoneurons in the left motor column relative to that in the right [$F(4,23) = 5.04$, $p < 0.005$]. Unilateral injection

of saporin into the left vastus medialis resulted in a 21% reduction in the relative number of motoneurons compared with that of untreated animals (LSD, $p < 0.009$). Treatment with testosterone or minocycline did not prevent this reduction (overall average of 23% reduced; LSDs, $p < .016$ compared to untreated animals); the reduced relative number of motoneurons within the quadriceps range did not differ across saporin groups [$F(2,15) = .74$, ns]. Treatment with minocycline in intact animals had no effect on motoneuron number (LSD, ns).

Motoneuron Morphometry—Injection of BHRP into the left vastus lateralis successfully labeled ipsilateral quadriceps motoneurons in all groups (Fig. 10). The dendritic arbor of quadriceps motoneurons was strictly unilateral, with extensive ramification along the ventrolateral margins of the gray matter and in the lateral funiculus, as well as throughout the ventral horn. An average of $37.75 (\pm 3.08)$ motoneurons per animal was labeled with BHRP, and this did not vary across groups [$F(4,24) = 1.79$, ns].

Dendritic Length. After saporin-induced motoneuron death, surviving neighboring quadriceps motoneurons underwent marked dendritic atrophy (Fig. 11). Dendritic length was decreased by 64% in saporin-injected animals compared to that of untreated animals [LSD, $p < .0001$; overall test for the effect of group on dendritic length $F(4,23) = 15.75$, $p < .0001$]. However, whereas dendritic length in testosterone-treated saporin animals was also shorter than that of untreated animals (LSD, $p < .002$), treatment with testosterone attenuated induced dendritic atrophy, with dendritic length being reduced on average by only 40%. Compared with saporin animals given no supplemental testosterone, testosterone-treated saporin animals had dendritic lengths that were 65% longer (LSD, $p < .04$).

Minocycline treatment did not prevent dendritic atrophy after partial motoneuron depletion. Dendritic length in minocycline-treated saporin animals was decreased by 79% compared to that of untreated animals [LSD, $p < .0001$]. Interestingly, treatment with minocycline in intact animals also resulted in reduced dendritic lengths (25% compared to untreated animals; LSD, $p < .05$).

Dendritic Distribution. Dendritic length was non-uniform across radial bins, and a repeated measures ANOVA revealed a significant effect of radial location [$F(11,253) = 10.10$, $p < .0001$; Fig. 12]. Consistent with the results in the dendritic length analysis, there was also a significant effect of group [$F(4,253) = 18.60$, $p < .0001$]. Reductions in dendritic length occurred throughout the radial distribution, ranging from 38% (180° to 240°) to 79% (60° to 120°) in saporin-injected animals compared with untreated animals [$F(1,99) = 26.19$, $p < .0007$]. Treatment with testosterone in saporin animals attenuated these reductions, ranging from 26% (300° to 360°) to 52% (120° to 180°) compared with untreated animals [$F(1,99) = 12.15$, $p < .007$]. Dendritic lengths per bin in testosterone-treated saporin animals were longer than those of saporin-injected animals [$F(1,110) = 6.63$, $p < .03$] throughout most of the radial distribution, with increases ranging from 8% (240° to 300°) to 139% (60° to 120°).

Dendritic distribution in minocycline-treated saporin animals did not differ from that of saporin only animals [$F(1,110) = 4.03$, ns], showing reductions in dendritic length

throughout the radial distribution, ranging from 41% (180° to 240°) to 92% (0° to 60°) compared with untreated animals [$F(1,99) = 70.67, p < .0001$]. Finally, consistent with what was found for total dendritic length, treatment with minocycline in intact animals resulted in reduced dendritic lengths throughout the radial distribution, ranging from no change (180° to 240°) to 49% (0° to 60°) compared with untreated animals [$F(1,99) = 31.43, p < .0003$].

Dendritic Extent.: In agreement with the nonuniform dendritic distribution of quadriceps motoneurons apparent in Fig. 12, radial dendritic extent differed across bins, and a repeated-measures ANOVA revealed a significant effect of location [$F(11,253) = 13.75, p < .0001$]. However, radial dendritic extent did not differ across groups [$F(4,253) = 2.23, ns$]. Rostrocaudal dendritic extent also did not differ across groups [$F(4,23) = 2.56, ns$], spanning an overall average of $3753.0 \pm 404.1 \mu\text{m}$.

DISCUSSION

Surviving motoneurons respond to the death of neighboring motoneurons with marked dendritic atrophy, and treatment with testosterone is neuroprotective, attenuating this induced dendritic atrophy (Fargo and Sengelaub, 2004a,b, 2007; Little et al., 2009; Wilson et al., 2009). Following injury, activated microglia contribute to the inflammatory immune response, release damaging reactive oxygen species, and phagocytose cellular debris or even dendritic branches (Gehrmann et al., 1993; Rappert et al., 2004; Loane and Byrnes, 2010; Neher et al., 2012; Kettenmann et al., 2013); this microglia response is hormone sensitive, and treatment with testosterone significantly reduces the number of microglia (Barreto et al., 2007). In the present study, in parallel with the induced dendritic atrophy and its mediation by testosterone, we observed a marked increase in the number of activated microglia following partial motoneuron depletion, and treatment with testosterone attenuated this upregulation. To directly test the hypothesis that activation of microglia after partial motoneuron depletion was responsible for the induced dendritic atrophy in surviving motoneurons, we inhibited microglial activation with minocycline. Minocycline treatment did not attenuate induced dendritic atrophy, suggesting that microglia are not the primary drivers of dendritic atrophy; in fact, reduction of microglial activation by minocycline treatment resulted in dendritic atrophy in intact animals. Together, these findings indicate that the neuroprotective effects of testosterone on dendritic arbors following motoneuron death are not due to the inhibition of microglial activation, and that microglial activity contribute to the normal maintenance of dendritic arbors.

Effectiveness of Saporin Injections

Consistent with our previous findings (Fargo and Sengelaub, 2004a,b, 2007; Little et al., 2009; Wilson et al., 2009; Cai et al., 2017), saporin injection into the vastus medialis significantly decreased muscle weight and reducing the number of motoneurons in the quadriceps pool by approximately 22% in the saporin-injected groups across both of the current studies. This saporin-induced death of motoneurons accounts for the elevation in microglia observed in Expt. 1, as well as the dendritic atrophy in surviving motoneurons observed in Expt. 2. Intramuscular injection of saporin had no effect on the adjacent, un-injected vastus lateralis muscle and there were no differences in the number of labeled

motoneurons following injection of BHRP into the vastus lateralis, important considerations for interpreting the effects seen in surviving motoneurons in Expt. 2.

Also consistent with previous findings (Little et al., 2009; Wilson et al., 2009), treatment with testosterone had no effect on saporin-induced decreases in weight in the vastus medialis muscle, nor did it prevent saporin-induced motoneuron death. Thus, the beneficial effects of testosterone treatment on the morphology of neighboring surviving motoneurons observed in Expt. 2 cannot be attributed to a hormone-mediated attenuation of the ability of saporin to kill motoneurons. Testosterone treatment also had no effect on the weights of non-saporin-injected muscles and thus differences in the morphology of surviving motoneurons cannot be ascribed to differences resulting from the degree of peripheral damage.

Similarly, minocycline treatment had no effect on muscle weight, neither preventing saporin-induced decreases in weight in the vastus medialis muscle, nor altering the weight of uninjected muscles. Thus, the reductions in dendritic length we observed in intact animals treated with minocycline in Expt. 2 are independent of target muscle size.

As expected, treatment with minocycline had no effect on motoneuron number, neither preventing reduction after saporin injection, nor reducing it in intact animals. The lack of effect in intact animals is particularly important- partial motoneuron depletion results in induced dendritic atrophy in surviving motoneurons (e.g., after saporin injection), and thus, the reduction in dendritic length we observed in intact animals treated with minocycline in Expt. 2 cannot be attributed to the death of motoneurons.

Activation of Microglia

As predicted, saporin-induced death of motoneurons resulted in significant increases in the total number, and more importantly, the relative number of activated microglia in the area of the quadriceps motor pool. However, the upregulation of microglia was brief- although both the total number and relative number of activated microglia were greatly increased at one week after saporin injection, by three weeks these had decreased to levels that did not differ from those of untreated animals. Given that atrophy in surviving quadriceps motoneuron dendrites occurs linearly over a four week period after induced motoneuron death (Coons et al., 2009), this transient upregulation is not likely responsible for the sustained period of progressive dendritic atrophy. Thus, the hypothesis that activation of microglia after partial motoneuron depletion is responsible for the induced dendritic atrophy in surviving motoneurons is likely incorrect. This conclusion is strongly supported by the results of Expt. 2- despite an inhibition of microglial activation, dendritic atrophy still resulted from partial motoneuron depletion.

Similar to the findings of Barreto et al. (2007), treatment with testosterone reduced the number of activated microglia in the area of the quadriceps motor pool after saporin-induced motoneuron death. This effect could be mediated by a direct effect of testosterone on microglia, as androgen receptor expression in microglia increases following chemical or physical lesion to neural tissue (Garcia-Ovejero et al., 2002). This upregulation of androgen receptors following injury may provide for a transient increased responsiveness of microglia to androgens following injury. Androgens could act to decrease reactive gliosis by affecting

the initial stages of microglia activation or down-regulate pro-inflammatory mediators that prolong glial activation and maintain the expression of activated microglial phenotypes (Garcia-Segura and Melcangi, 2006). Alternatively, testosterone treatment could reduce the survival of reactive cells, ultimately terminating reactive gliosis (Jones et al., 1997b; White et al., 1998; Dihne et al., 2001). Finally, testosterone treatment could potentially promote a shift from the activated phenotypes to the ramified form (Barreto et al., 2007). Testosterone could exert its effects through signaling pathways that do not directly or immediately involve transactivation of genomic DNA (Foecking et al., 2015). Receptor independent effects of steroid hormones have been identified [e.g., antioxidant effects through downregulating the formation of reactive oxygen species (Mosquera et al., 2014; Wang et al., 2006) or inhibiting lipid peroxidation reactions (Singh et al., 2006)].

Testosterone can also be metabolized into estradiol via the enzyme aromatase, allowing it to indirectly signal via the estrogen receptor. We have previously demonstrated that treatment with estradiol after partial motoneuron depletion attenuates induced dendritic atrophy in surviving motoneurons to a degree comparable to that achieved with testosterone, and that this neuroprotection requires action through steroid hormone receptors (Cai et al., 2017). Interestingly, aromatase expression increases in astrocytes, but not microglia, following injury, allowing for para- or autocrine estrogen signaling as a response to the injury to mitigate the harm of excessive inflammation and gliosis (Garcia-Segura et al., 1999). Astrocytes also show an upregulation in estrogen receptor expression following stab wound to the brain (Garcia-Ovejero et al., 2002), and treatment with estradiol attenuates gliosis (Vegeto et al., 2003; Barreto et al., 2007). During gliosis, astrocytes normally secrete inflammatory cytokines and other signaling factors that contribute to microglial chemotaxis and shift in function (Garcia-Ovejero et al., 2005; Garcia-Segura and Melcangi, 2006; Biber et al., 2007). This suggests an indirect pathway for testosterone treatment to indirectly reduce microglial recruitment via an attenuation of paracrine astrocyte signaling.

Causes and Protection from Dendritic Atrophy

Consistent with our previous studies (Little et al., 2009; Wilson et al., 2009; Cai et al., 2017), saporin-induced motoneuron death resulted in a pronounced dendritic atrophy in surviving nearby quadriceps motoneurons. We have previously speculated that the atrophy observed in remaining motoneurons could be a result of an increased microglial activation in the injury site, resulting in collateral damage through dendritic loss (Cai et al., 2017). As discussed above, the current studies indicate that this hypothesis is likely incorrect. Alternatively, we have also previously speculated that the death of motoneurons could result in the release of toxins [e.g., inflammatory cytokines (IL-6, IL-1 β , TNF- α), purines (ATP), glutamate, and matrix metalloproteinases (MMPs)] into the extracellular space, in close proximity to the dendrites of the surviving motoneurons resulting in direct damage (Fargo and Sengelaub, 2004a). Such local changes in the cellular microenvironment would be consistent with the general atrophy seen throughout the dendritic distribution.

As reported previously (Little et al., 2009; Wilson et al., 2009; Cai et al., 2017), treatment with testosterone attenuated induced atrophy of quadriceps motoneuron dendrites. Treatment with testosterone produces a wide array of neuroprotective and neurotherapeutic effects

(Fargo et al., 2009a). For example, the expression of multiple proteins involved in structural plasticity are androgen-sensitive, including enhanced expression of the cytoskeletal proteins β -tubulin (Jones and Oblinger, 1994; Jones et al., 1999b; Brown et al., 2001; Matsumoto et al., 1994) and GAP-43 (Coers et al., 2002; Jones et al., 1997a), and elevated neuritin mRNA expression (Fargo et al., 2008). Similarly, proteins thought to be involved in neuroprotection are also regulated by androgens, including increased expression of heat shock protein (Zhang et al., 2004; Tetzlaff et al., 2007) and proteins with antioxidant functions (e.g., catalase; Ahlbom et al., 2001). Androgens are also thought to be involved with the activation of signaling pathways involved in neuroprotection (e.g., MAPK/ERK, Pike et al., 2008). Testosterone mediates the central glial response (Jones et al., 1999a) and attenuates synaptic stripping seen after motoneuron injury, preserving central input to the motoneurons (Jones et al., 1997a). In the present study, testosterone treatment reduced the microglial response after induced motoneuron death. However, given that our evidence suggests that elevated levels of activated microglia are not responsible for producing dendritic atrophy, it is unlikely that a testosterone-mediated down-regulation of microglia results in the attenuation of that atrophy.

Alternatively, testosterone could have a neuroprotective effect, sparing motoneuron dendrites from atrophy induced by partial motoneuron depletion. We have previously speculated that one such effect could be mediated through androgen receptors at the target musculature (Cai et al., 2017). Support for the target muscle as the site of androgenic action comes from a variety of sources (Foecking et al., 2015). For example, motoneuron dendritic morphology is regulated by androgens acting at the target musculature (Huguenard et al., 2011). Androgen action at the target musculature has also been shown to regulate BDNF expression in motoneurons (Verhovshek and Sengelaub, 2013), and we have demonstrated that BDNF is critical in maintaining normal dendritic length in motoneurons (Verhovshek and Sengelaub, 2010). Thus, it is possible that testosterone's neuroprotective effects following partial motoneuron depletion are due to testosterone binding at the target muscle, signaling to the innervating motoneurons to produce BDNF or some other trophic factor, which protects motoneurons from dendrite atrophy. This attenuation of dendritic retraction would thus secondarily result in reduced numbers of neuropathologically activated microglia.

Microglia and Dendritic Maintenance

If activation of microglia was responsible for the induced dendritic atrophy in surviving motoneurons, inhibition of microglial activation following saporin injection should have protected those motoneurons from dendritic atrophy. Contrary to this hypothesis, minocycline treatment of saporin-injected animals had no protective effect, and dendrites in surviving motoneurons were reduced in length to levels even below (although not statistically different from) those of saporin-injected animals. While microglia may not be the primary driving force behind dendritic atrophy following motoneuron death, these results suggest that activated microglia may be required to maintain normal dendritic length. In their basal state, microglia monitor the health of dendritic spines and synapses by using their highly motile processes to probe the pre- and post-synaptic regions (Trapp et al., 2007; Wake et al., 2009; Tremblay et al., 2011; Salter and Beggs, 2014). Visualization using electron microscopy and the presence of neurotransmitter receptors in glial processes indicate that as microglia engulf a synapse, they receive cues from the neurons, synaptic cleft, or

extracellular space that indicate whether the synapse is either ‘survival worthy’ or a target for pruning (Pocock and Kettenmann, 2007; Wake et al., 2009; Tremblay et al., 2011). In addition to eliminating synapses, microglia may also contribute to plasticity through the formation and strengthening of new synapses as well. For example, transgenic microglial depletion produces marked reductions in both spine formation *and* elimination in the hippocampus and motor cortex of mice, which were associated with deficits in learning a new motor pattern (Parkhurst et al., 2013). Microglia are also known to produce a variety of secreted factors (interleukin-4, interleukin-10, brain-derived neurotrophic factor) that have been implicated as neuroprotective (Gorski et al., 2003; Yang et al., 2004; Zhao et al., 2006; Parrish et al., 2007). It is possible that minocycline treatment attenuates the production of some or all of these trophic factors, resulting in dendritic retraction due to reduced trophic signaling required to maintain normal dendritic length.

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 (Fargo and Sengelaub, 2004b; Goldstein and Sengelaub, 1994; Hebbeler et al., 2002; Kurz et al., 1991) are affected by hormone levels. Thus, in the present study, we believe that the differences we observed across treatment groups reflect dendritic atrophy in surviving motoneurons in saporin-injected animals, which is attenuated by treatment with testosterone. The possibility that confounds arising from saporin injection or minocycline treatment 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 across groups were observed. Therefore, we believe that the dendritic labeling across groups was comparable and that the shorter dendritic lengths we observed in saporin groups and intact animals treated with minocycline reflect dendritic atrophy.

CONCLUSIONS

Treatment with androgens (Fargo et al., 2009a; Foecking et al., 2015) promotes a wide array of neuroprotective and neurotherapeutic effects. In this study we assessed a potential mechanism through which testosterone might attenuate secondary atrophy in surviving neighboring motoneurons by regulating the microglia response to induced motoneuron death. Our findings indicate that the neuroprotective effects of testosterone on dendritic arbors following motoneuron death are not due to the inhibition of microglial activation. However, our results do provide evidence that microglial activity contributes to the normal maintenance of dendritic arbors. Understanding the cellular and molecular mechanisms that operate in normal and injured neurons is likely to provide important insights for developing therapeutic interventions for nervous system trauma and neurodegenerative diseases.

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Figure 1.

(A) The area of interest for microglial counts (gray rectangle) was determined using the location of the contralaterally BHRP-labeled quadriceps motoneurons (indicated by stars). For each animal, the rostrocaudal limits (dashed lines) of the quadriceps motoneuron distribution were superimposed contralaterally over the corresponding immunohistochemically-stained sections. (B) Brightfield digital micrograph of a horizontal section through the lumbar spinal cord, showing BHRP-labeled motoneurons following BHRP injection into the right vastus lateralis muscle. Scale bars = 500 μm .

Figure 2.

Weights of the vastus medialis muscles corrected by body weight in untreated animals and saporin-injected animals that either received no further treatment (SAP) or were treated with testosterone (SAP+T) at one week and three weeks after saporin injection. Gray bars represent weights from the right (R) and left (L) sides in untreated animals. Black bars represent weights from the untreated contralateral (right) side of the quadriceps muscle, and white bars represent weights from the saporin injected (left) side of the quadriceps muscle of saporin-injected animals. Saporin injection reduced the weight of the vastus medialis muscle; treatment with testosterone had no effect on muscle weight. Bar heights represent means $\pm$ SEM. * indicates significantly different from untreated animals.

Figure 3.

Numbers of quadriceps motoneurons in untreated animals and saporin-injected animals that either received no further treatment (SAP) or were treated with testosterone (SAP+T) at one week and three weeks after saporin injection, expressed as a ratio of motoneuron number ipsilateral to the saporin-injected muscle relative to that on the untreated side. Saporin killed approximately 25% of the ipsilateral quadriceps motoneurons, regardless of subsequent treatment. Bar heights represent means $\pm$ SEM. * indicates significantly different from untreated animals.

Figure 4.

Brightfield digital micrographs of horizontal sections through the lumbar spinal cord, showing quadriceps motoneuron cell bodies stained with neutral red and microglia immunostained with Iba-1 in an untreated animal (A), and saporin-injected animals with either no further treatment (B) or treated with testosterone (C) at one week after saporin injection into the vastus medialis muscle. Examples of microglial phenotypes, classified as monitoring (D) or activated, including primed (E), reactive (F) and amoeboid (G) forms. Scale bars: A-C = 100 μ m; D-G = 20 μ m.

Figure 5.

(A) Total number of microglia and (B) numbers of activated microglial phenotypes relative to the monitoring form following partial depletion of quadriceps motoneurons with saporin (SAP). At one week following the death of quadriceps motoneurons, both the total number of microglia and the relative number of activated forms were significantly elevated over levels found in untreated animals. Treatment with testosterone (T) attenuated these increases. At three weeks after saporin injection, both the total number of microglia and the relative number of activated microglia in the saporin-injected and testosterone-treated saporin animals had decreased to levels that did not differ from those of untreated animals. Bar heights represent means $\pm$ SEM. * denotes significantly different from untreated

animals; † denotes significantly different from saporin animals that received no further treatment.

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Figure 6.

Brightfield digital micrographs of horizontal sections through the lumbar spinal cord, showing quadriceps motoneuron cell bodies stained with neutral red and microglia immunostained with Iba-1 in an untreated animal (A), and saporin-injected animals with either no further treatment (B) or treated with minocycline (C) at one week after saporin injection into the vastus medialis muscle. Scale bar = 100 μ m.

Figure 7.

(A) Densities of total microglia and (B) activated microglial phenotypes relative to the monitoring form following partial depletion of quadriceps motoneurons with saporin (SAP). At one week following the death of quadriceps motoneurons, the densities of both total microglia and the relative number of activated forms were significantly elevated over levels found in untreated animals. Treatment with minocycline (SAP+MINO) attenuated these increases. Bar heights represent means $\pm$ SEM. * denotes significantly different from untreated animals; † denotes significantly different from saporin animals that received no further treatment.

Figure 8.

Weights of the vastus medialis (A) and vastus lateralis (B) muscles corrected by body weight in untreated animals, and saporin-injected animals that either received no further treatment (SAP) or were treated with testosterone (SAP+T), or minocycline (SAP+MINO). Gray bars represent weights from the right (R) and left (L) sides in untreated animals or intact animals treated with minocycline (intact+MINO). Black bars represent weights from the untreated contralateral (right) side of the quadriceps muscle, and white bars represent weights from the saporin injected (left) side of the quadriceps muscle of saporin-injected animals. Saporin injection reduced the weight of the vastus medialis muscle but had no effect on the adjacent vastus lateralis muscle. Treatment with testosterone or minocycline had no effect on muscle

weight. Bar heights represent means $\pm$ SEM. * indicates significantly different from untreated animals.

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Figure 9.

Numbers of quadriceps motoneurons in untreated animals and saporin-injected animals that either received no further treatment (SAP) or were treated with testosterone (SAP+T) or minocycline (SAP+MINO), and intact animals treated with minocycline (intact+MINO) at four weeks after saporin injection, expressed as a ratio of motoneuron number ipsilateral to the saporin-injected muscle relative to that on the untreated side. Saporin killed approximately 23% of the ipsilateral quadriceps motoneurons, regardless of subsequent treatment. Treatment with minocycline had no effect on motoneuron number. Bar heights represent means $\pm$ SEM. * indicates significantly different from untreated animals.

Figure 10.

Darkfield digital micrographs of transverse hemisections through the lumbar spinal cords and computer-generated reconstructions BHRP-labeled somata and processes of an untreated animal (A,B), and saporin-injected animals with either no further treatment (C,D), or treated with testosterone (E,F), an intact animal treated with minocycline (G,H), and a saporin-injected animal treated with minocycline (I,J) after BHRP injection into the left vastus lateralis muscle. Computer-generated composites of BHRP labeling 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 .

Figure 11.

Dendritic lengths of quadriceps motoneurons in untreated animals and saporin-injected animals that either received no further treatment (SAP) or were treated with testosterone (SAP+T) or minocycline (SAP+MINO), and intact animals treated with minocycline (intact +MINO). Following saporin-induced motoneuron death, surviving neighboring motoneurons lost almost 64% of their dendritic length. Treatment with testosterone attenuated this dendritic atrophy, but inhibition of microglial activation with minocycline had no effect. Treatment of intact animals with minocycline reduced dendritic length by 25%. Bar heights represent means $\pm$ SEM. * indicates significantly different from untreated animals. † indicates significantly different from untreated saporin-injected animals.

Figure 12.

Inset: Drawing of spinal gray matter divided into radial sectors for measure of quadriceps motoneuron dendritic distribution. Length per radial bin of quadriceps dendrites in untreated animals (black bars) and saporin-injected animals that either received no further treatment (SAP, white bars) or were treated with testosterone (SAP+T, light gray bars) or minocycline (SAP+MINO, gray bars), and intact animals treated with minocycline (intact+MINO, darkest gray bars). For graphic purposes, dendritic length measures have been collapsed into 6 bins of 60° each. Quadriceps motoneuron dendritic arbors display a non-uniform distribution, with the majority of the arbor located between 300° and 120°. Following saporin-induced motoneuron death, surviving neighboring motoneurons had reduced dendritic length throughout the radial distribution. Treatment with testosterone attenuated this reduction, but inhibition of microglial activation with minocycline had no effect. Treatment of intact animals with minocycline reduced dendritic length throughout the radial distribution. Bar heights represent means $\pm$ SEM. * indicates significantly different from untreated animals. † indicates significantly different from untreated saporin-injected animals.