

TESTOSTERONE AS A NEUROPROTECTIVE/NEUROTHERAPEUTIC AGENT  
FOLLOWING PARTIAL NEURONAL DEPLETION

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## Dedication

This thesis and the work contained herein are dedicated to my dear wife, Susan Elaine Fargo. This process was as difficult for her as it was for me, even more so in many ways, and it is impossible for me to imagine that I would have been able to accomplish this goal without her constant love and support.

Thank you.

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## Chapter 1.

### Introduction and Background

Motoneuron loss is a significant medical problem, and can result from disease processes or from traumatic injuries. For example, amyotrophic lateral sclerosis (ALS) is a fatal disease characterized by the rapidly progressive loss of motoneurons, affecting 2-3 people per 100,000 population per year, with over 6000 new diagnoses made every year in the United States alone. While ALS is the most prevalent, other diseases are also characterized by progressive loss of motoneurons, including the motor neuron diseases (MNDs) and spinal muscular atrophies (SMAs). Traumatic spinal cord injuries are even more prevalent: in the United States, more than 10,000 people per year survive a spinal cord injury. Of these, around 45% suffer from spinal motoneuron lesions, and this number rises to around 95% for those with lumbar or sacral injuries (Doherty et al., 2002).

Many treatment strategies have been developed to enhance neuronal survival and regeneration in disease and following traumatic injuries (Tsai and Tator, 2005). Some of these involve macro-scale physical changes in the environment of the injury or diseased tissue, such as by the grafting of peripheral nerve sheaths (e.g., Storer et al., 2002) or the transplantation of supportive cells (Xiang et al., 2005). The observation that these kinds of treatments also produce molecular-level changes to the environment has led to attempts at treating injured or diseased tissue directly with various regeneration associated molecules (Makwana and Raivich, 2005). By far, the class of molecules that has received the most attention in this capacity is the neurotrophic factors; and in fact, neurotrophic factors appear to have dramatic effects in preventing neuron death and promoting neuron regeneration (e.g., Blits et al., 2004; Clatterbuck et al., 1994; Kishino et al., 1997; Novikov et al., 2000; Wu et al., 2003; Yang and Arnold, 2000b; Yang et al.,



2004). The principal drawback of using neurotrophic factors therapeutically is that most of them will not cross the blood-brain barrier, meaning that they have limited efficacy when simply administered systemically (Wu, 2005).

Gonadal steroids also exhibit a wide array of neuroprotective and neurotherapeutic effects, and in contrast to neurotrophic factors, readily cross the blood-brain barrier and (Henderson and Reynolds, 2002; Jones, 1993; Jones et al., 2001; Woolley and Cohen, 2002). For example, testosterone treatment accelerates both axon regeneration and functional recovery following axotomy of spinal or cranial motoneurons (Jones et al., 2001). After crush axotomy of hamster facial motoneurons, treatment with exogenous testosterone accelerates the recovery of motor function (Kujawa and Jones, 1990; Kujawa et al., 1989); this effect appears to be due to an acceleration in the rate of facial motoneuron axon regeneration, which is also increased with exogenous testosterone treatment (Kujawa et al., 1991). Interestingly, the timing of testosterone treatment is critical, as only immediate treatment with testosterone has been shown to accelerate functional recovery or axon regeneration (Kujawa et al., 1991; Kujawa and Jones, 1990; Tanzer et al., 2000; Tanzer and Jones, 2004), and longer treatment times result in greater accelerations (Kujawa et al., 1991; Tanzer et al., 2000).

I have begun to extend findings of the neuroprotective/neurotherapeutic effects of testosterone to dendrites. The dendritic arbor is the most striking morphological feature of many neurons, accounting for up to 97% of the surface area of a neuron (Cameron et al., 1985), and accommodating up to 100,000 synaptic contacts (Burke, 1990; Ulfhake and Cullheim, 1988). Dendritic morphology is intimately connected to neuronal identity and function. For example, motoneurons innervating different muscles have distinct

dendritic morphologies (Cameron et al., 1985; Cullheim et al., 1987b; Furicchia and Goshgarian, 1987; Ritz et al., 1992; Schoenen, 1982; Ulfhake and Kellerth, 1981), and within a muscle motoneurons innervating fast vs. slow muscle fibers are associated with different dendritic morphologies (Cullheim et al., 1987a). Dendritic structure is also tied to the electrophysiological response properties of neurons. Differences in morphology are reflected electrophysiologically, even between different types of neurons within a particular structure (Grudt and Perl, 2002; Lu et al., 2001; Rumberger et al., 1998); and dendritic morphology has a direct effect on the electrophysiological responses of the cell (Mainen and Sejnowski, 1996; Vetter et al., 2001).

Several forms of neural injury are known to impact neuronal morphology, including deafferentation and axotomy. Deafferentation often results in dendritic retraction and atrophy of deafferented neurons (e.g., Brown et al., 1979; Caceres and Steward, 1983; Somogyi et al., 1987; Wellman and Sengelaub, 1995). However, deafferentation of motoneurons has also been shown to result in several other morphological responses, including biphasic expansion and retraction of dendrites (Bernstein and Standler, 1983) and the development of distinct size classes in previously homogeneous populations (Bernstein et al., 1984), and even appears to be permissive of dendritic growth in certain cases (Bury et al., 2000; Jones and Schallert, 1994). Most studies of the effects of axotomy on neuronal morphology have also reported dendritic atrophy (e.g., Brännström et al., 1992; Naumann et al., 1992; O'Hanlon and Lowrie, 1995; Sumner and Watson, 1971; Yawo, 1987; but see Rose and Odlozinski, 1998). However, this is not the only response reported; for example, Standler and Bernstein

(1982) described a cyclic pattern of repeated degeneration and regeneration of the dendritic arbor following crush axotomy.

I have been examining the therapeutic effects of testosterone on dendritic morphology in an injury model using the spinal nucleus of the bulbocavernosus [SNB; also known as the dorsomedial nucleus or DM (Schröder, 1980); see Figure 1.1]. The SNB is a small, discrete, well-characterized population of sexually dimorphic motoneurons located in the lumbar spinal cord of the rat. The SNB is bilaterally organized, and in males, motoneurons from each half of the nucleus innervate the ipsilateral bulbocavernosus (BC) and levator ani (LA) muscles (BC/LA), as well as the external anal sphincter (Breedlove and Arnold, 1980; McKenna and Nadelhaft, 1986; Schröder, 1980). This neuromuscular system is involved in erectile functions and is critically important for male sexual behavior (Hart and Melese-D'Hospital, 1983; Sachs, 1982).

The large somata of SNB motoneurons support broad dendritic arbors which extend throughout a large volume of the surrounding neuropil (Kurz et al., 1986). Owing to their medial location, this creates a large region of bilateral dendritic overlap (Goldstein et al., 1993; Kurz et al., 1986; Rose and Collins, 1985; see Figure 1.2). Both SNB motoneurons and their target muscles are sensitive to testosterone manipulations (Breedlove and Arnold, 1981; Kurz et al., 1986; Matsumoto, 1997; Wainman and Shipounoff, 1941). Androgens maintain a wide variety of SNB motoneuron characteristics, including the morphology of somata (Breedlove and Arnold, 1981) and dendrites (Kurz et al., 1986), the number and size of gap junction plaques (Matsumoto et al., 1988), actin and tubulin mRNA expression (Matsumoto et al., 1993; Matsumoto et

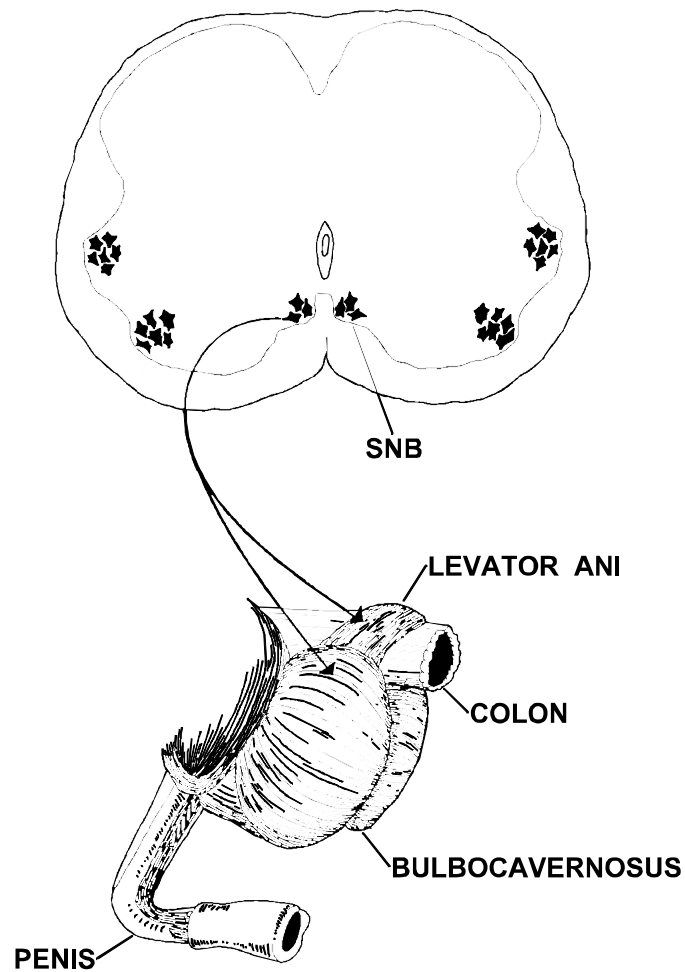


FIGURE 1.1

Schematic representation of the spinal nucleus of the bulbocavernosus (SNB) and its projections to the muscles of the perineum in the male rat. Note the medial placement of the nucleus within the spinal cord. Although it is not clear in this graphic, individual SNB motoneurons can project to either the BC or the LA, but not both.

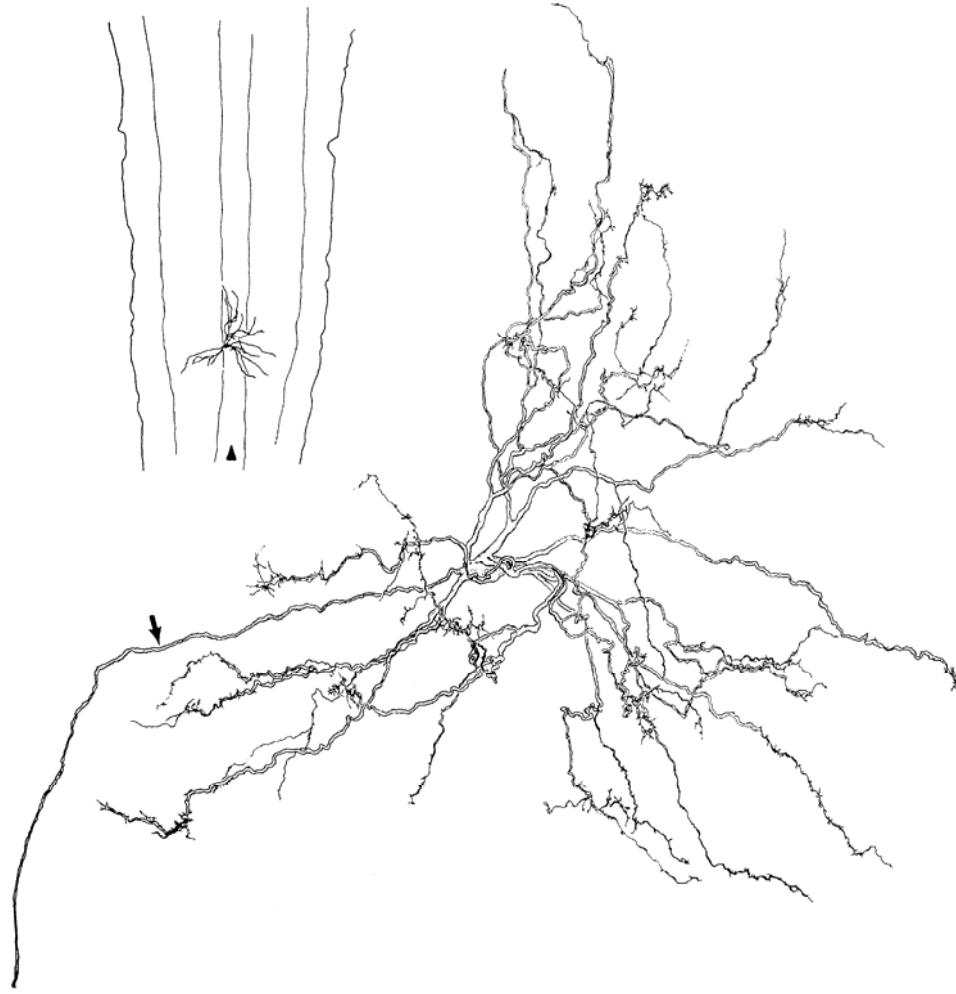


FIGURE 1.2

Camera lucida reconstruction of a single left-side SNB motoneuron after intracellular fill with HRP. Arrow indicates axon.

Inset: location of this motoneuron within the lumbosacral spinal cord. Arrowhead indicates midline. Note the prominent arborization of dendrites across the midline.

From Sasaki and Arnold (1991).

al., 1992; Matsumoto et al., 1994), and levels of synaptic input (Leedy et al., 1987; Matsumoto et al., 1988). Testosterone also modulates several important biochemicals in the SNB, including ciliary neurotrophic factor receptor  $\alpha$  (Forger et al., 1998), calcitonin gene-related peptide (Monks et al., 1999; Popper and Micevych, 1989; Popper and Micevych, 1990), N-cadherin (Monks et al., 2001; Monks and Watson, 2001), Bcl-2 (Zup and Forger, 2002), and BDNF (Yang et al., 2004). These features make the SNB an excellent system in which to study the therapeutic effects of testosterone on dendritic morphology following injury. The SNB is also particularly exciting as a model system from a clinical perspective. Approximately 80% of spinal cord injury patients are male, and 60% are under 30 years old, making the effects of injury to the male perineal neuromuscular system especially relevant.

Lesions of the CNS typically result in cell death in affected neuronal populations. Additionally, neuron loss can lead to morphological changes in other neuronal populations related by proximity and/or connectivity (e.g., Fargo and Sengelaub, 2004a, 2004b; Wellman and Sengelaub, 1991, 1995). Presumably, some loss of motor function after injury is mediated by this secondary cellular atrophy. Additionally, absent the ability to replace dead neurons, surviving motoneurons must be the basis for recovering function lost due to the primary cell death. Therefore, learning how to prevent or reverse neuronal atrophy secondary to neuron depletion is an important goal. This forms the rationale for this dissertation.

### Previous Studies

I have conducted two previous studies relevant to the present thesis. In the first (Fargo and Sengelaub, 2004a), I showed that unilateral motoneuron depletion leads to

atrophy of motoneurons on the contralateral side of the spinal cord. Moreover, I showed that testosterone treatment protected motoneurons from that atrophy, thus demonstrating a neuroprotective effect of testosterone in a motoneuron injury model. To induce unilateral motoneuron death, I injected a toxin consisting of saporin conjugated to cholera toxin B subunit (CTB-saporin) into the right BC and LA muscles of adult male Sprague Dawley rats. When injected into the perineal muscles, CTB-saporin interacts with GM<sub>1</sub> ganglioside (Abe and Norton, 1974) on SNB nerve terminals, causing the neurons to endocytose the toxin, which is then retrogradely transported to their somata in the spinal cord. Saporin is a type 1 ribosome inactivating protein; it irreversibly inactivates ribosomes, thereby halting protein synthesis and leading to cell death (Bergamaschi et al., 1996; Stirpe, 2004; Stirpe et al., 1992; Stirpe et al., 1983). CTB-saporin has previously been used to kill sympathetic preganglionic neurons after injection into the superior cervical ganglion (Llewellyn-Smith et al., 1999) and facial motoneurons after injection into the facial nerve (Llewellyn-Smith et al., 2000). Unilateral CTB-saporin injection into the perineal musculature results in laterally specific motoneuron depletion: approximately 60% of SNB motoneurons ipsilateral to the injected muscle die, while the number of motoneurons contralateral to the injection remains unchanged. Some animals treated with CTB-saporin also underwent a testosterone manipulation. They were castrated 6 weeks prior to injection with CTB-saporin, then at the time of injection were given subcutaneous testosterone-filled Silastic implants designed to produce plasma testosterone titers in the high-normal physiological range (3.18 mm outer diameter, 1.57 mm inner diameter, 45 mm long; Smith et al., 1977).

Four weeks later, I prepared the contralateral SNB motoneurons for visualization. I injected HRP conjugated to the cholera toxin B subunit (CTB-HRP; a retrogradely transported tracer) into the left BC muscle. Two days later, animals were killed and spinal cords were removed and processed for CTB-HRP visualization with a modification of Mesulam's (1982) tetramethyl-benzidine protocol. SNB motoneurons contralateral to the CTB-saporin injection were viewed under darkfield illumination, and reconstructed in three dimensions. Motoneuron depletion resulted in significant dendritic and somal atrophy in contralateral motoneurons. The effect was particularly large in dendrites, which lost over 60% of their normal length. However, testosterone treatment prevented this outcome for dendrites: animals injected with CTB-saporin and treated with testosterone had normal dendritic length. As SNB motoneurons are known to receive trophic support from their target muscles (al-Shamma and Arnold, 1995; Rand and Breedlove, 1995; Yang and Arnold, 2000a), it is worth noting that the BC and LA muscles were hypertrophied in testosterone-manipulated animals. It is therefore possible that testosterone acts at the target muscle, which then provides enough target support to the surviving motoneurons to overcome the effects of unilateral depletion.

The testosterone manipulation in this study was relatively complex, and left room for several possible interpretations as to which aspect of the manipulation was protecting dendritic morphology. The protective element might have been the castration-induced state of regression the SNB motoneurons were in at the time of the CTB-saporin injections, the active regrowth process induced in the SNBs of castrated animals by testosterone reintroduction, or the high-normal systemic levels of testosterone produced by the implants.



This issue was resolved in the second study (Fargo and Sengelaub, 2004b). Three groups of animals were compared: normal controls, animals given unilateral intramuscular injections of CTB-saporin, and animals both injected with CTB-saporin and treated with exogenous testosterone. However, the testosterone treatment differed from the previous study: testosterone-treated animals were both castrated and given testosterone implants simultaneously on the day on CTB-saporin injection. This design eliminates the possibility that motoneuron morphology could be affected by either the regression caused by castration or the regrowth caused by testosterone reintroduction. Consistent with the results of our first study (Fargo and Sengelaub, 2004a), the dendrites of testosterone-treated males were of normal length despite injection with CTB-saporin. Thus, the protective effect of testosterone treatment following unilateral motoneuron depletion is not dependent on castration-induced regression or the active regrowth caused by delayed testosterone reintroduction. Instead, the exogenous testosterone itself has a protective effect. As in the previous study, the BC and LA muscles of testosterone-treated animals were hypertrophied.

These studies present an intriguing possibility. For the reasons outlined in the background section, the SNB is an especially appealing system in which to study the functional and morphological effects of neuronal depletion and testosterone treatment. It is possible that these studies could form the basis of a model preparation with which to examine these phenomena. Several important basic questions need to be answered about the effects observed in these studies: 1) can similar effects of depletion and testosterone treatment can be demonstrated between motoneuron groups innervating muscles on the same side of the body; 2) does motoneuron depletion affect the electrophysiological

response properties of contralateral SNB motoneurons, and can testosterone treatment restore them to normal; 3) do the effects of CTB-saporin injection depend on a direct effect of the exogenous toxin; and 4) can the protective effect of testosterone be due the activity of its estrogenic metabolite? These questions form the basis of the experiments in this dissertation.

### Organization of the Thesis

The thesis is divided into seven chapters. In this first chapter, I introduce the background and significance of the dissertation work, including my previous studies that form the basis for the dissertation. Several methods and techniques are used in more than one experiment in the dissertation; in the second chapter I describe these in detail and justify their use. Chapters 3 through 6 form the bulk of the thesis. In these chapters I describe the individual experiments that form the dissertation. In each chapter I give the rationale for the experiment, explain the experimental design (including explaining and justifying divergences from the common techniques described in Chapter 2), describe the results of the experiment, and interpret and discuss those results. In Chapter 7, I discuss broadly the significance of the major findings of the dissertation.

## Chapter 2.

### Experimental Methods and Techniques

Several of the methods and techniques in this dissertation are used in more than one experiment. Instead of describing them and the justifications for their use in multiple chapters, I will describe them and explain the general reasons for their use here. In subsequent chapters I will refer back to these descriptions. In cases where these techniques have special implications for a specific experiment, I will explain those in the chapter containing that experiment.

### Animals

All animals were adult male Sprague Dawley rats obtained from Harlan (Indianapolis, Indiana), and were approximately 100 days old at the beginning of each experiment. Animals were maintained on a 12:12-hour light:dark cycle, with *ad libitum* access to food and water.

### Saporin Injection

Saporin is a type 1 ribosome inactivating protein derived from the plant *saponaria officinalis*, commonly known as soapwort (Stirpe et al., 1983). Ribosome inactivating proteins work by irreversibly inactivating ribosomes, thereby halting protein synthesis and leading to cell death. On its own, however, saporin is relatively innocuous, as it is almost completely incapable of crossing cell membranes to enter cells (Stirpe, 2004). When conjugated to other molecules, though, saporin can be used as a very effective targeted toxin. Typically, saporin is conjugated to another molecule that confers upon it the ability to enter a specific class of cells, usually a cell that expresses a receptor for the conjugated molecule, while bypassing other cell types (Lappi and Wiley, 2004). In the current experiments, I use a form of saporin conjugated to the B subunit of cholera toxin and mixed with distilled water to produce a 0.1% (w/v) solution. This form of saporin is

commonly referred to as CTB-saporin. CTB-saporin enters cells that express the cell surface ganglioside GM<sub>1</sub>. In the perineal musculature, the nerve terminals of SNB motoneurons express GM<sub>1</sub>, so CTB-saporin injected into the perineal muscles is actively endocytosed by SNB motoneurons. This method results in the death of approximately 60% of the SNB motoneurons ipsilateral to the injected muscles, but none of the contralateral SNB motoneurons. The principal advantages of intramuscular injection of CTB-saporin are that it kills motoneurons a) in a muscle-specific manner, and b) without the complications of mechanical injury to the surrounding neuropil.

Animals were placed under general anesthesia by putting them in an induction chamber containing ether-soaked cotton pads until they no longer responded to deep pain. Animals were then placed in the supine position, and a cone containing ether-soaked cotton pads placed adjacent to their snouts in order to maintain areflexia to noxious stimuli. A midline incision was then made in the ventral scrotal wall, and blunt dissection was performed to expose the right side BC/LA muscle complex. I then injected a 1 µl bolus of 0.1% saporin solution into the belly of the right BC muscle using a 10 µl Hamilton syringe. In order to minimize leakage, the syringe was slowly and carefully withdrawn after the injection. I then repeated this procedure to inject another 1 µl of the saporin solution into the right LA muscle. This exposes most of the motoneurons in the ipsilateral SNB to the toxin (presumably, most of the SNB motoneurons that survive this procedure project to the external anal sphincter). The scrotum was then closed with 9-mm AutoClips brand stainless steel wound clips (MikRon Precision, Inc.; Gardena, CA), and after any other surgical procedures were completed, the animal was allowed to recover from anesthesia.

## Castration

Animals were placed under general anesthesia by putting them in an induction chamber containing ether-soaked cotton pads until they no longer responded to deep pain. Animals were then placed in the supine position, and a cone containing ether-soaked cotton pads placed adjacent to their snouts in order to maintain areflexia to noxious stimuli. A small midline incision was then made in the ventral scrotal wall. Forceps were used to grasp a portion of the tunica immediately overlying the right *cauda epididymidis*, and a small portion of it was dissected away, creating an opening. The entire right testicle and epididymis were extruded through this opening, along with a length of the ductus deferens and as much of the adipose tissue of the spermatic cord as was possible without risking traumatic injury to the animal. A ligature of silk cord was then made around the ductus deferens and the blood vessels (and any remaining adipose tissue) of the spermatic cord, and these were cut distal to the ligature. The detached testicle, epididymis, and adipose tissue were discarded. The ligated ductus deferens and spermatic cord were then tucked back into the scrotum, and the process was repeated on the left side using the same incision in the scrotum. The scrotum was then closed, and after any additional surgical procedures appropriate to the experiment were performed, the animal was allowed to recover from anesthesia.

Castration is used to remove the body's major source of circulating testosterone. (Although the adrenal gland does produce some testosterone, its amount is vanishingly small and its functional significance is negligible.) When used alone, the experimental purpose of castration is to determine the effect of removing testosterone from the model organism. When used in conjunction with hormone replacement (see below), the

experimental purpose is to reduce variability in testosterone levels, both between animals and within animals.<sup>1</sup>

### Testosterone Implants

Testosterone replacement was given in the form of Silastic implants. Silastic implants were formed from 55-mm lengths of Silastic brand silicone tubing with a 1.57 mm inner diameter and a 3.18 mm outer diameter (Dow Corning; Midland, MI). A 5 mm wooden dowel was lodged into one end of the Silastic tube. Using a custom made tamper, I then filled the tube with crystalline testosterone (4-androsten-17 $\beta$ -ol-3-one; Steraloids, Newport, RI), leaving 5 mm at the other end empty. Another 5 mm dowel was then lodged into the empty end. To remove testosterone from the outside of the implants, they were then cleaned with a Kimwipe (Kimberly-Clark, Neenah, WI) moistened with absolute ethanol. The ends were then sealed with Silastic brand silicone Type A medical adhesive (Dow Corning); one coat was applied and allowed to cure overnight, then a second coat was applied and allowed to cure until implantation (at least overnight). The finished implants had a length of 45 mm of testosterone apposed to the surface of the Silastic tubing.

Implantation was accomplished under ether anesthesia, in conjunction with either castration or saporin injection or both, as described above. After the other procedure or procedures were completed, the animal was placed in the prostrate position and a small incision was made into the skin between the scapulae. The implant was then placed under the skin through this incision. The incision was closed with 9-mm AutoClips brand stainless steel wound clips, and the animal was allowed to recover from anesthesia.

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<sup>1</sup> In intact adult male rats, testosterone levels fluctuate constantly throughout the day, ranging between 0.3 and 8.9 ng/ml plasma (Södersten et al., 1983), with a daily average of  $2.36 \pm 0.25$  (Mean  $\pm$  SEM) ng/ml plasma (Damassa et al., 1977).

Silastic silicone tubing is a permeable material, and testosterone passes through it at a constant rate. Thus, implants like these can be used to deliver relatively steady levels of testosterone to the body, and the amount of testosterone delivered can be controlled by varying the length of the implant. 45-mm implants, like those used in these experiments, produce levels of testosterone in the bloodstream toward the high end of the normal range (Smith et al., 1977).

### Visualization of Spinal Motoneurons

#### HRP injection

SNB motoneurons were retrogradely labeled for visualization with horseradish peroxidase (HRP) conjugated to the B subunit of cholera toxin. As mentioned in the section describing saporin injections above, the cholera toxin B subunit interacts with the GM<sub>1</sub> ganglioside on the nerve terminals of SNB motoneurons to induce endocytosis of their conjugate. While free HRP is readily taken up by nerve terminals and transported to the somata and primary dendrites of motoneurons, HRP conjugated to cholera toxin B subunit provides a much more complete cellular fill and enables the visualization of even distal dendritic branches (for an example, see Figure 3.4); because of this, it has become the standard label used in anatomical studies of SNB motoneurons. In the current experiments, I use a form of HRP conjugated to the B subunit of cholera toxin and mixed with distilled water to produce a 0.2% (w/v) solution. This form of HRP is commonly referred to as CT-HRP, CTB-HRP, BHRP, or  $\beta$ HRP. However, for the sake of readability I will refer to it throughout this thesis simply as HRP (when I refer to unconjugated HRP, I will specifically identify it as such).



Previous experiments (Fargo and Sengelaub, 2004a, 2004b; Kurz et al., 1986) have established that two days is an optimal length of time to wait between HRP injection and sacrifice in the SNB/BC system, allowing for the visualization of cell bodies and distal processes; therefore HRP injection occurred two days prior to sacrifice. After placing the animals under ether anesthesia, injections proceeded as described above for saporin injections. However, there were two very important differences between saporin injections and HRP injections. First, HRP injections were made into only the BC muscle. The reason for this is that the vast majority of the previous literature on the anatomy of the somata and dendrites of SNB motoneurons have utilized BC-projecting motoneurons (e.g, Fargo and Sengelaub, 2004a, 2004b; Goldstein et al., 1993; Kurz et al., 1986; Yang et al., 2004). Therefore, in order to facilitate comparison with previous studies, I too used BC-projecting motoneurons as my model. The second important difference between the saporin injections and the HRP injections is that the HRP injections were made into the *left side* of the muscle complex. Note that this means that the motoneurons visualized for anatomical study are *contralateral* to the motoneurons that were killed by saporin injection. This is consistent with previous studies (Fargo and Sengelaub, 2004a, 2004b; Goldstein et al., 1993). Each animal received a 0.5  $\mu$ l bolus of 0.2% HRP solution into the left side BC muscle.

#### Spinal cord removal and processing

Two days after HRP injection, animals were killed with an overdose of urethane. Animals were weighed and given an i.p. injection of urethane dissolved in distilled water for a total dose of 0.25 g urethane per 100 g body weight. If the animal did not reach areflexia to deep pain within approximately 10 to 15 minutes, an additional 1 ml i.p.

injection of the urethane solution was given. Rarely, an animal required yet another 1 ml i.p. injection to reach areflexia to deep pain.

Once animals reached the appropriate level of anesthesia, they were placed in the supine position and the skin was cut across the ventrum just caudal to the sternum. The abdominal wall was then lifted and cut just caudal to the sternum, with care taken to avoid damaging the liver. The diaphragm was then cut, with care taken to avoid damaging the heart or lungs. The ribcage was then cut along both sides and reflected to expose the heart. A cannula was placed in the left ventricle and the right atrium was cut. Animals were then perfused through the heart with saline solution fed by a gravity pump. This was followed by perfusion with cold fixative (1% paraformaldehyde/1.25% glutaraldehyde).

Animals were then placed in the prostrate position and an incision of approximately 10 cm was made into the skin of the back directly over the spine. The skin was then spread and a portion of the spinal column approximately 6 to 7 cm long and containing the points of articulation for the most caudal 3 to 4 ribs was removed. This portion of the spinal column was then trimmed until it contained the point of articulation of only the final rib and was only 3 to 4 cm long. This method reliably conserves the portion of the spinal cord that contains the SNB. The spinal cord was then extracted by inserting a dowel into the caudal end of the trimmed portion of the spinal column and pushing.

Animals were then placed back in the supine position, and an incision was made in the midline of the scrotum. Blunt dissection was performed to expose the perineal

muscles. The BC/LA muscle complex was then removed and dissected into its component muscles, which were weighed separately.

Extracted spinal cords were then postfixed in cold 1% paraformaldehyde/1.25% glutaraldehyde for 5 hours, and stored in cold sucrose phosphate buffer (10% w/v, pH 7.4) overnight for cryoprotection. Spinal roots were then removed, leaving only a small stump for each, and spinal cords were trimmed to include only the 5th and 6th lumbar segments and the 1st sacral segment, the portion of the cord that contains the SNB. Spinal cords were then embedded in a gelatin block, which was subsequently fixed and cryoprotected overnight in a cold solution of 10% sucrose phosphate buffer and fixative. The embedded spinal cords were then frozen-sectioned transversely at 40  $\mu\text{m}$ . Sections were collected into four alternating series, so that the distance between the centers of consecutive sections in each series was 160  $\mu\text{m}$ .

One series was immediately mounted onto slides coated with porcine gelatin and allowed to dry for three days. They were then stained with thionin and coverslipped with Permount. This series was used for cell counting (see below). The other three series were processed for HRP visualization using tetramethyl benzidine as a chromagen, which makes the HRP label appear black (or exceedingly dark blue). These series were then mounted onto porcine gel-coated slides, allowed to dry for three days, counterstained with thionin, and coverslipped with Permount. These three series were used for motoneuron morphometry (see below).

#### Cell counting

SNB motoneurons are large, multipolar neurons. They stain darkly with thionin, reside in a discrete area in the spinal cord, and are easily recognizable (see Figure 2.1).

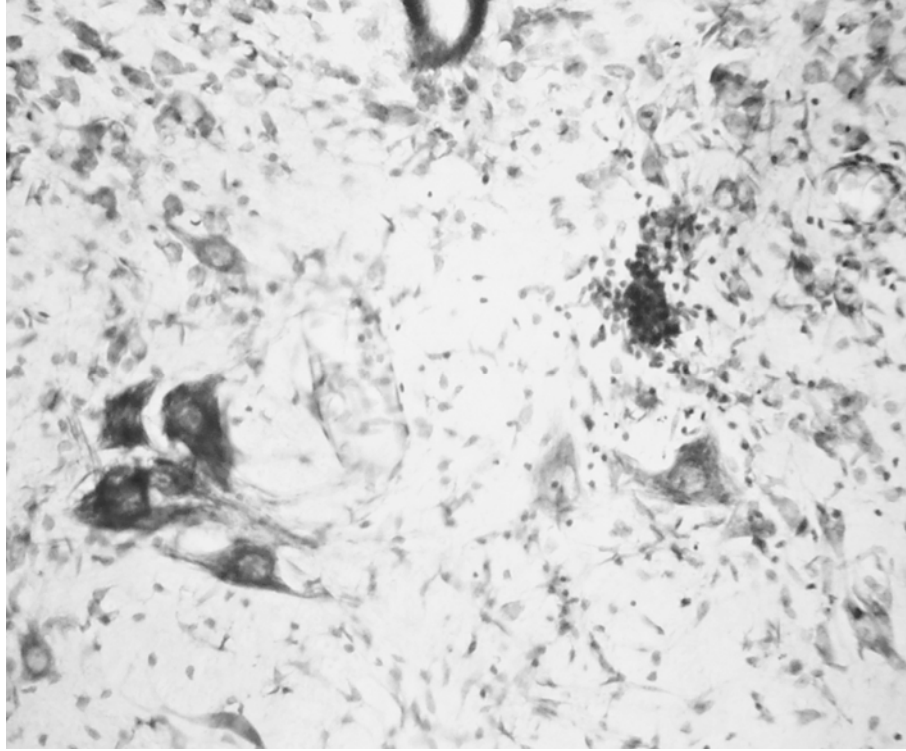


FIGURE 2.1

Photomicrograph of SNB motoneurons 6 days after unilateral injection of saporin into the right BC and LA muscles; depletion of motoneurons in the right half of the nucleus is apparent, as is concomitant gliosis.

Because HRP labeling can obscure thionin stained motoneurons, and thus result in spuriously low cell counts, motoneurons were counted only in the series that was *not* processed for HRP visualization. To correct for the fact that motoneurons were counted in only every fourth section, motoneuron counts were multiplied by four to determine a total for each animal. Counting every motoneuron that has a piece contained in the section being examined would lead to an artificially inflated cell count, because motoneurons that extend into consecutive sections would contribute to cell counts more than once. To correct for this, motoneuron profiles that extended through the top of the section were not counted; I only counted motoneurons that were wholly contained within the section or that extended through the bottom of the section. Motoneurons were counted separately on both sides of the spinal cord, under brightfield illumination and at a final magnification of 500X.

#### Motoneuron morphometry

Measurements of motoneuron morphology were taken in the three series processed for HRP visualization. HRP-labeled SNB motoneurons were counted in every section of each of these series in exactly the same way that thionin-stained motoneurons were counted in the other series.

Soma size. Cross-sectional motoneuron area was determined by using a video-based computerized morphometry system (Stereo Investigator; MicroBrightField, Colchester, VT) to trace the profiles of HRP-filled SNB somata, under brightfield illumination and at a final magnification of 780X. A minimum of approximately 20 motoneurons per animal were traced, encompassing at least one entire series. Sometimes it was necessary to trace SNB motoneuron profiles from more than one series in order to

collect the minimum number. In these cases, somata were traced for the entire rostrocaudal extent of *each* series used. This was done to avoid any possible size bias that might have been introduced by tracing motoneurons preferentially from one end of the nucleus.<sup>2</sup> Soma areas within each animal were then averaged for statistical analysis. In addition, individual soma areas were sorted into 100- $\mu\text{m}^2$  bins, and group differences in their frequency distributions were assessed by  $\chi^2$  analyses.<sup>3</sup>

Dendrites. For each animal, measures of dendritic length, distribution, and extent were all taken from a single series processed for HRP visualization. This series was determined by picking the series that had the middle number of HRP-labeled SNB motoneurons (i.e., neither the series with the greatest number nor the series with the least number was used). In cases in which two series had the same number of HRP-labeled motoneurons, a qualitative evaluation of the amount of dendritic arbor was made, and the series that would yield the most conservative result was used, thus minimizing the chances of producing a spurious effect.

*Length.* Using the predetermined series, and beginning with the most rostral section in which HRP-labeled processes were visible, I used a computer-based morphometry system (Neurolucida; MicroBrightField) connected to a camera lucida to trace the dendritic arbor in three dimensions. Tracings were made under darkfield illumination at a final magnification of 250X, and were collected from every other section in the series (centers were 320  $\mu\text{m}$  apart) through the entire rostrocaudal extent of the nucleus. The lengths of all the traced processes were then summed, multiplied by two

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<sup>2</sup> SNB motoneurons near the rostral end of the nucleus tend to have larger somata than those near the caudal end. Moreover, SNB motoneurons from the rostral vs. caudal ends of the nucleus have been shown to have differential sensitivity to some experimental manipulations.

<sup>3</sup> In some cases, extreme low and high bins were grouped in order to avoid expected values of less than one, which would invalidate conclusions drawn from the  $\chi^2$  test.

to correct for sampling, and divided by the number of HRP-labeled motoneurons in the traced series. This method assesses dendritic length throughout the dendritic arbor of the entire nucleus, and yields an estimate of dendritic length per motoneuron. While this estimate does not represent a measurement of actual total dendritic length for the nucleus (Kurz et al., 1991), it has proven to be a sensitive and reliable indicator of changes in dendritic morphology during normal development (Goldstein et al., 1993; Goldstein et al., 1990; Goldstein and Sengelaub, 1993), in response to hormonal manipulations (Burke et al., 1999; Burke et al., 1997; Forger and Breedlove, 1987; Goldstein et al., 1990; Goldstein and Sengelaub, 1993; Goldstein and Sengelaub, 1994; Hebbeler and Sengelaub, 2003; Hebbeler et al., 2001; Hebbeler et al., 2002; Kurz et al., 1991; Kurz et al., 1986), after changes in dendritic interactions (Goldstein et al., 1993) and afferent input (Hebbeler and Sengelaub, 2003; Hebbeler et al., 2002; Kalb, 1994), and after induced motoneuron death (Fargo and Sengelaub, 2004a, 2004b). In keeping with the established literature, for the sake of simplicity, this measure is referred to throughout this thesis as either “dendritic length” or “arbor per cell”.

*Distribution.* A set of radial axes was then applied to the transverse aspect of the computerized tracings. These axes originated at the central canal and divided the spinal cord into 12 radial bins of 30° each. Dendritic length per motoneuron was then determined for each radial bin by summing the lengths within that bin, multiplying by two to correct for sampling, and dividing the result by the number of HRP-labeled motoneurons in the traced series. This measure, then, is essentially the same as the dendritic length measure, but apportioned into radial bins. This method provides a sensitive measure of dendritic redistribution in response to changes in dendritic

interactions (Goldstein et al., 1993) and afferent input (Hebbeler and Sengelaub, 2003; Hebbeler et al., 2002), and after induced motoneuron death (Fargo and Sengelaub, 2004a, 2004b). In keeping with the previous literature, this measure is referred to throughout this thesis as “dendritic length per bin” or “arbor per cell per bin”.

*Extent.* Using the same set of axes, I then measured in each radial bin the simple linear distance between the central canal and the most distal HRP-labeled process. This provides an estimate of the maximal extent of the dendritic field in the mediolateral plane, as well as providing another measure of dendritic distribution. The extent of the dendritic arbor was also measured rostrocaudally. I counted the number of sections that contained HRP label in the series used for morphometry. As the centers of these sections are 160  $\mu\text{m}$  apart, I multiplied this count by 160  $\mu\text{m}$  to arrive at an estimate of the linear distance between the most rostral and most caudal HRP-labeled processes. For the sake of simplicity, these measures are referred to throughout this thesis simply as “radial dendritic extent” and “rostrocaudal dendritic extent”.



## Chapter 3.

Does Testosterone Protect against Motoneuron Atrophy  
Induced by Depletion of Ipsilateral Motoneurons?

The SNB is a medial motor nucleus, and extends dendrites throughout a large area of the surrounding neuropil, including prominent projections across the midline where their dendritic arbors overlap with those of their contralateral counterparts (Collins et al., 1991; Goldstein et al., 1993; Kurz et al., 1986; Rose and Collins, 1985; Sasaki and Arnold, 1991). This is the normal pattern for medial motor nuclei. However, the majority of spinal motoneurons lie in the lateral motor columns, and their dendrites do not overlap with their contralateral counterparts. Instead, they overlap with other motoneuron populations that also reside in the lateral motor columns but project to different (ipsilateral) muscles. Overlapping dendritic fields provide several possible ways in which motoneuron populations can influence each other: they offer a discrete area for common afferents to project to, they allow for dendro-dendritic chemical signaling, they are likely nourished and modulated by a common population of glia (in some cases even by the same individual glial cells), and specifically in the case of the SNB, they allow for electrical signaling between sub-populations via gap junctions (Coleman and Sengelaub, 2002).

Overlapping dendritic fields, then, represent a promising potential object of study in examining the neuroprotective effects of testosterone following motoneuron depletion. Based only on the observations made in the previous studies (Fargo and Sengelaub, 2004a, 2004b), one cannot rule out the possibility that the atrophy induced by motoneuron depletion is limited to medial motoneurons that innervate the same muscle on the other side of the body and have contralaterally overlapping dendritic arbors. While this would still be interesting from a basic neuroscience perspective, the beneficial effects of testosterone treatment would be more broadly applicable if they extend to motoneuron

populations that reside on the same side of the spinal cord and have ipsilaterally overlapping dendritic arbors. Thus, this experiment is designed to unilaterally deplete a population of motoneurons, and examine the effects of testosterone treatment on remaining *ipsilateral* motoneurons that share its dendritic field.

Because the majority of motoneurons lie in the lateral motor columns, the ideal endpoint is to test these effects in lateral motor column populations. However, given that we know that the SNB responds to contralateral motoneuron depletion with atrophy and is particularly sensitive to testosterone, a logical first step is to attempt an ipsilateral experiment in the SNB. Therefore, I have addressed the question by unilaterally depleting SNB motoneurons, then examining the morphology of surviving SNB motoneurons that reside on the same side of the spinal cord but project to a different muscle. The SNB contains three distinct populations of motoneurons that can be defined by the muscles they innervate: those that project to the BC, those that project to the LA, and those that project to the external anal sphincter (EAS; McKenna and Nadelhaft, 1986; Schröder, 1980). If the atrophy observed in the previous studies is related to overlapping dendritic arbors, then I ought to be able to be able to kill motoneurons in one of the SNB populations and observe atrophy in motoneurons of another SNB population on the same side of the spinal cord. More importantly, if the overlapping dendritic arbors provide testosterone with a substrate on which to exert its beneficial effects, testosterone treatment should prevent this atrophy.

#### METHODS AND DESIGN

The majority of SNB motoneurons project to the BC, with most of the remainder projecting to the LA, and a few projecting to the EAS (Kurz et al., 1990; McKenna and

Nadelhaft, 1986; Schröder, 1980). In my previous experiments, I have ensured the death of a large majority of SNB motoneurons by injecting saporin into both the BC and LA. However, killing both BC- and LA-projecting SNB motoneurons leaves only the EAS-projecting motoneurons to visualize on the ipsilateral side of the body. I decided against attempting to visualize EAS-projecting motoneurons for three reasons. First, it is technically difficult to make HRP injections reliably into the EAS. Second, the small number of neurons that project to the EAS would reduce the stability of my morphological measures, thus decreasing the sensitivity of the experiment and necessitating the use of more animals for no theoretical gain over using the other motoneuron populations. Third and most importantly, the morphology of EAS-projecting motoneurons is not affected by testosterone manipulation (Collins et al., 1992). In fact, I am unaware of any previous studies that have used morphological measures of dendrites of HRP-labeled EAS-projecting motoneurons as dependent variables.

This left the option of killing only BC- or LA-projecting motoneurons, then examining the morphology of motoneurons in the remaining subpopulation. Both BC- and LA-projecting motoneurons have been used to study the effects of steroid hormone treatment on spinal motoneurons (e.g, Kurz et al., 1990; Kurz et al., 1986). LA-projecting motoneurons were chosen for morphometry because there are more BC-projecting motoneurons (Kurz et al., 1990), thus offering the opportunity to kill a large portion of SNB motoneurons (those that project to the BC) while still leaving enough alive (those that project to the LA) to collect reliable measures.

Adult male Sprague Dawley rats were used. Saporin injections were made into only the BC muscle on the left side of the animal. Saporin injections were made

according to the method described in Chapter 2, with the exception that a 1  $\mu$ l injection was made into the BC only, and no saporin injection was made into the LA. Coincident with saporin injection, some animals were also simultaneously castrated and given replacement testosterone according the methods described in Chapter 2 (saporin only  $n = 7$ , saporin and testosterone treatment  $n = 6$ ). A group of untreated intact normal males ( $n = 6$ ) and a group of untreated castrates ( $n = 4$ ) served as control groups. Four weeks after saporin injection and testosterone-treatment onset, I injected HRP into only the LA muscle on the left side (ipsilateral to the motoneuron depletion). The HRP injection was carried out according to the method described in Chapter 2, with the exception that the injection was made into the ipsilateral LA instead of the contralateral BC. After two additional days to allow for optimal HRP transport, animals were killed and the left side perineal musculature removed and weighed. Lumbosacral spinal cords were removed and processed for motoneuron counts and morphometry according to the methods described in Chapter 2.

## RESULTS

### Muscle Weights

Intramuscular injection of saporin into the BC muscle significantly reduced BC muscle weight [ $0.379 \pm 0.025$  g (mean  $\pm$  SEM) for saporin-injected animals compared to  $0.492 \pm 0.020$  g for normal controls, LSD,  $p < .001$ ; overall test for the effect of group on BC muscle weight  $F(3, 19) = 26.16$ ,  $p < .001$ ; see Figure 3.1, left]. Treatment with testosterone completely prevented this muscular atrophy ( $0.494 \pm 0.018$  g for saporin-injected animals treated with testosterone; compared to normal controls, LSD, *ns*). Consistent with the previous literature (Fargo and Sengelaub, 2004a; Wainman and

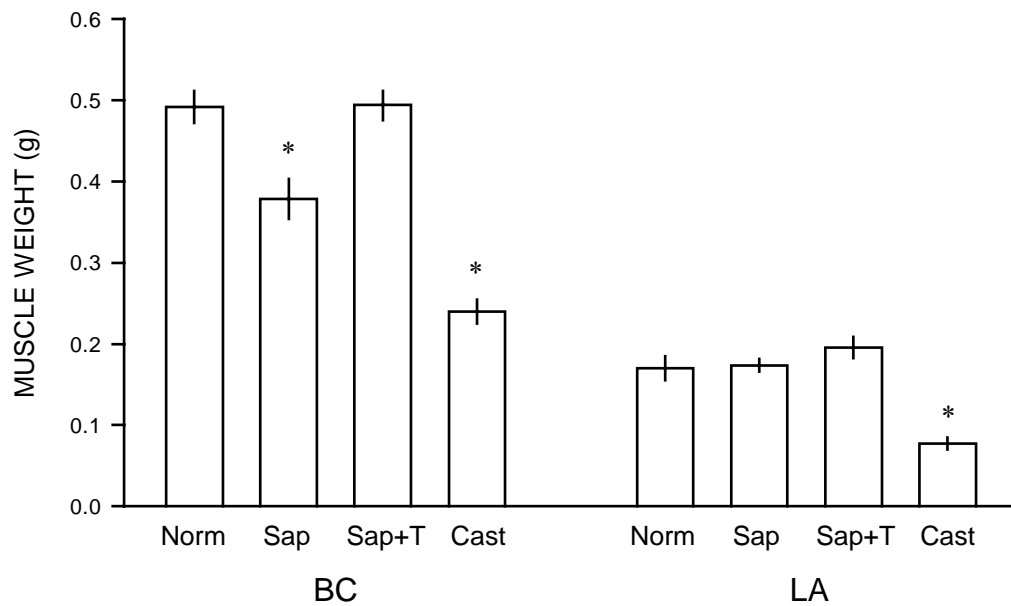


FIGURE 3.1

Unilateral BC (left) and LA (right) muscle weights for normal controls (Norm), untreated saporin-injected animals (Sap), saporin-injected animals treated with testosterone (Sap+T), and castrated controls (Cast). Castration decreased both BC and LA weight markedly. In the BC, saporin injection decreased muscle weight, but testosterone treatment completely prevented this. In the LA, neither saporin injection nor testosterone treatment significantly affected muscle weight. \* indicates significantly different from normal controls,  $p < .001$ . Bar heights represent means  $\pm$  SEM.

Shipounoff, 1941), castration significantly reduced BC muscle weight ( $0.240 \pm 0.015$  g for castrated controls; compared to normal controls, LSD,  $p < .001$ ).

LA muscle weight displayed a much different pattern. Consistent with the previous literature (Eisenberg et al., 1949; Fargo and Sengelaub, 2004a; Wainman and Shipounoff, 1941), castration significantly reduced LA muscle weight ( $0.078 \pm 0.007$  g for castrated controls compared to  $0.170 \pm 0.015$  g for normal controls, LSD,  $p < .001$ ; overall test for the effect of group on LA muscle weight  $F(3, 19) = 15.21, p < .001$ ; see Figure 3.1, right]. However, neither saporin injection into the BC nor testosterone treatment had a significant effect on LA muscle weight ( $0.174 \pm 0.008$  g for saporin-injected animals and  $0.195 \pm 0.013$  g for saporin-injected animals treated with testosterone; compared to normal controls, LSDs, *ns*).

### Cell Counts

Because lateral differences are irrelevant in this experiment, motoneuron counts were made only on the side of the animal that was injected with saporin. Injection of saporin into the BC muscle resulted in the death of over 40% of ipsilateral SNB motoneurons [ $61.71 \pm 10.14$  remaining motoneurons for saporin-injected animals compared to  $108.00 \pm 7.01$  for normal controls, LSD,  $p < .01$ ; overall test for the effect of group on motoneuron number  $F(3, 19) = 6.46, p < .01$ ; see Figure 3.2], and treatment with testosterone did not prevent this ( $56.17 \pm 10.30$  remaining motoneurons for saporin-injected animals treated with testosterone; compared to normal controls, LSD,  $p < .01$ ). Consistent with previous studies (Breedlove and Arnold, 1981; Fargo and Sengelaub, 2004a, 2004b), castration did not result in the death of SNB motoneurons ( $98.00 \pm 6.77$  remaining motoneurons for castrated controls; compared to normal controls, LSD, *ns*);

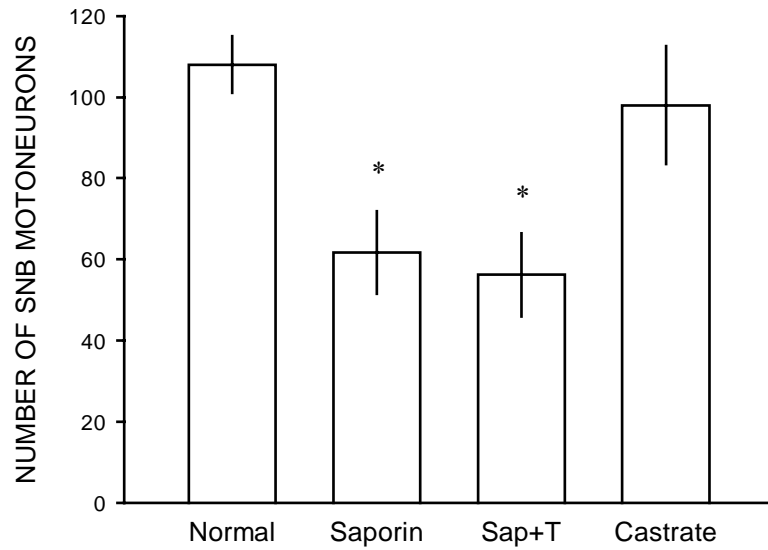


FIGURE 3.2

Number of surviving SNB motoneurons for normal controls, untreated saporin-injected animals, saporin-injected animals treated with testosterone, and castrated controls. Saporin injection into the BC muscle resulted in the death of approximately 40% of SNB motoneurons. Treatment with testosterone did not prevent this. Cell counts are unilateral, and were taken on the left side of the animal in every case. \* indicates significantly different from normal controls,  $p < .01$ . Bar heights represent means  $\pm$  SEM.



the numbers of SNB motoneurons in both castrated and normal controls were well within the normal range.

### Morphometry

#### Somata

Somata were traced for an average of  $20.13 \pm 0.89$  HRP-labeled motoneurons per animal, and this did not differ between groups [ $F(3, 19) = 0.47, ns$ ]. Soma area was an average of  $950.69 \pm 37.27 \mu\text{m}^2$ , and group differences did not reach significance [ $F(3, 19) = 2.07, ns$ ]. However, soma areas for castrated controls ( $763.88 \pm 73.93 \mu\text{m}^2$ ) were much smaller than for any other group, whereas saporin-injected animals, both untreated ( $991.61 \pm 56.65 \mu\text{m}^2$ ) and testosterone-treated ( $970.47 \pm 53.31 \mu\text{m}^2$ ), had soma areas very similar to those of normal controls ( $1007.33 \pm 90.18 \mu\text{m}^2$ ; see Figure 3.3).

$\chi^2$  analysis of individual soma areas yielded similar results (see Figure 3.4).

Neither saporin-injected animals nor saporin-injected animals treated with testosterone had frequency distributions that were different from that of normal controls [ $\chi^2(13) \leq 13.18, ns$ ]. In contrast, castration alone shifted the distribution of individual soma areas significantly toward the lower end of the spectrum [compared to normal controls,  $\chi^2(13) = 37.09, p < .001$ ; overall test for the effect of group on the distribution of individual soma areas  $\chi^2(39) = 79.34, p < .001$ ].

#### Dendrites

Injection of HRP into the left LA successfully labeled ipsilateral SNB motoneurons in a manner consistent with previous studies (Kurz et al., 1991). SNB motoneurons displayed their characteristic multipolar morphologies (Kurz et al., 1991; Kurz et al., 1986; Sasaki and Arnold, 1991), with dendritic arbors projecting

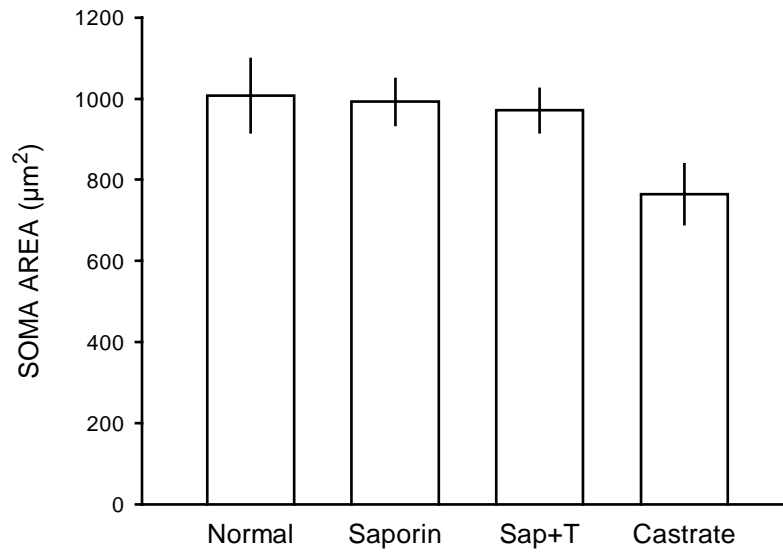


FIGURE 3.3

Cross-sectional soma areas of LA-projecting SNB motoneurons for normal controls, untreated saporin-injected animals, saporin-injected animals treated with testosterone, and castrated controls. No group differed significantly from normal (however, see the text regarding castrated controls). Bar heights represent means  $\pm$  SEM.

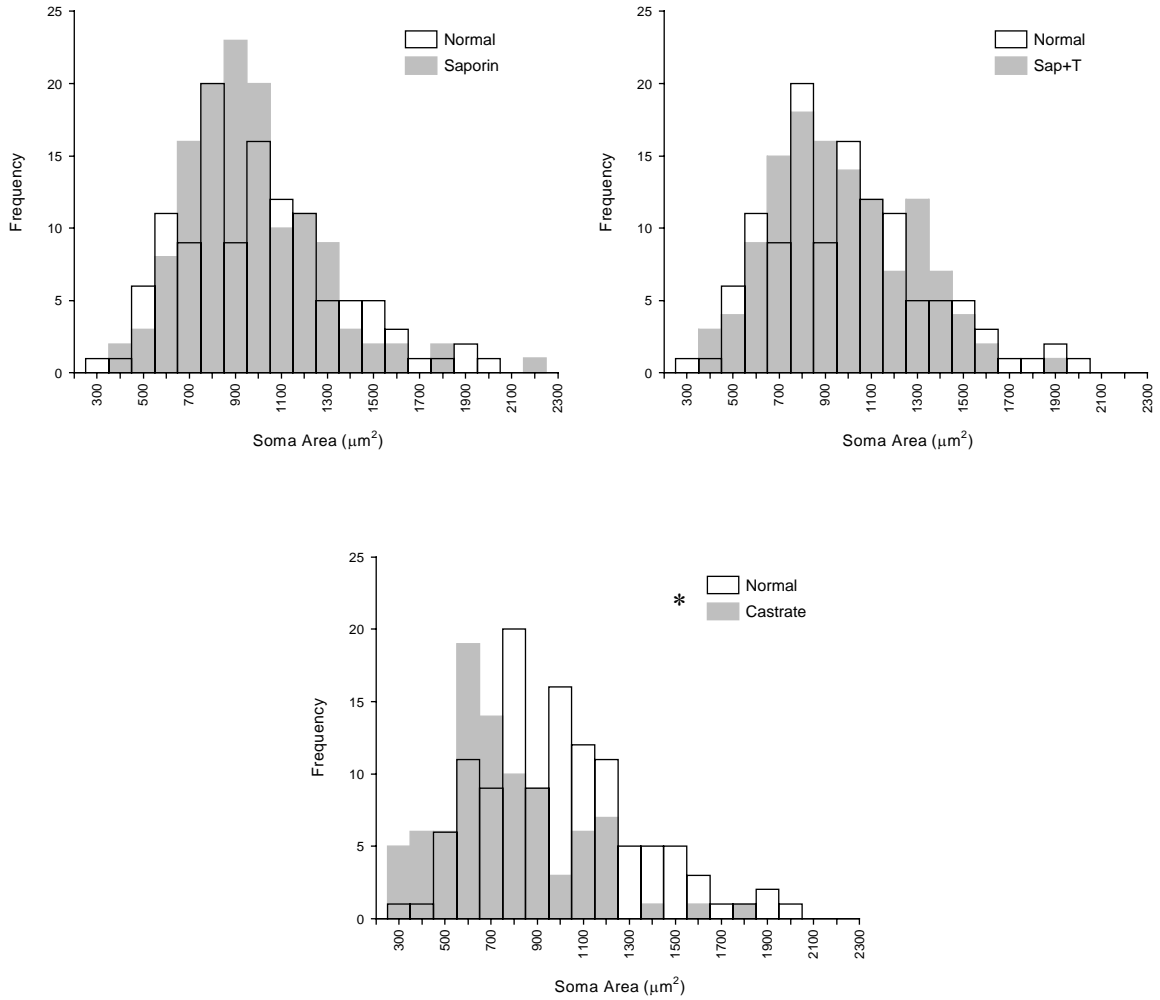


FIGURE 3.4

Frequency distributions of individual soma areas of LA-projecting SNB motoneurons for normal controls, untreated saporin-injected animals, saporin-injected animals treated with testosterone, and castrated controls. Only castrated controls had a frequency distribution different from that of normal controls, with castration shifting the distribution toward smaller somata. \* indicates significantly different from normal controls,  $p < .001$ .

ventrolaterally, dorsomedially, and across the midline into the area of the contralateral SNB (Figure 3.5). An average of  $35.13 \pm 2.41$  motoneurons were labeled with HRP, and this did not differ between groups [ $F(3, 19) = 1.59, ns$ ]. This is markedly fewer than the number of labeled motoneurons typically found when HRP is injected into the BC muscle (average of about 57 motoneurons; Fargo and Sengelaub, 2004a, 2004b), and reflects the fact that there are fewer LA-projecting than BC-projecting motoneurons in the SNB (Kurz et al., 1990).

Saporin treatment reduced dendritic length by almost 60% [ $3836.72 \pm 1114.31$   $\mu\text{m}$  for saporin-injected animals compared to  $9089.44 \pm 2065.54$   $\mu\text{m}$  for normal controls; LSD,  $p < .05$ ; overall test for the effect of group on dendritic length  $F(3, 19) = 3.40, p < .05$ ; see Figure 3.6]. Surprisingly, treatment of saporin-injected animals with testosterone completely failed to prevent this dendritic atrophy ( $4364.33 \pm 1034.88$   $\mu\text{m}$ ; compared to normal controls, LSD,  $p < .05$ ). Castrated controls also displayed significant dendritic atrophy ( $3746.82 \pm 582.94$   $\mu\text{m}$ ; compared to normal controls, LSD,  $p < .05$ ).

Dendritic length was nonuniform across radial bins, and repeated-measures ANOVA revealed a significant effect of radial location [ $F(11, 209) = 77.70, p < .001$ ]. Consistent with the results of the arbor per cell analysis, there was also a significant effect of group [ $F(3, 19) = 3.42, p < .05$ ]. Saporin injection led to reductions in arbor length of approximately 81% from  $0^\circ$  to  $30^\circ$ , 75% from  $30^\circ$  to  $60^\circ$ , 72% from  $60^\circ$  to  $90^\circ$ , 74% from  $90^\circ$  to  $120^\circ$ , 64% from  $120^\circ$  to  $150^\circ$ , 60% from  $150^\circ$  to  $180^\circ$ , 61% from  $180^\circ$  to  $210^\circ$ , 44% from  $210^\circ$  to  $240^\circ$ , 50% from  $240^\circ$  to  $270^\circ$ , 56% from  $270^\circ$  to  $300^\circ$ , 52% from  $300^\circ$  to  $330^\circ$ , and 70% from  $330^\circ$  to  $360^\circ$  (see Figure 3.7).

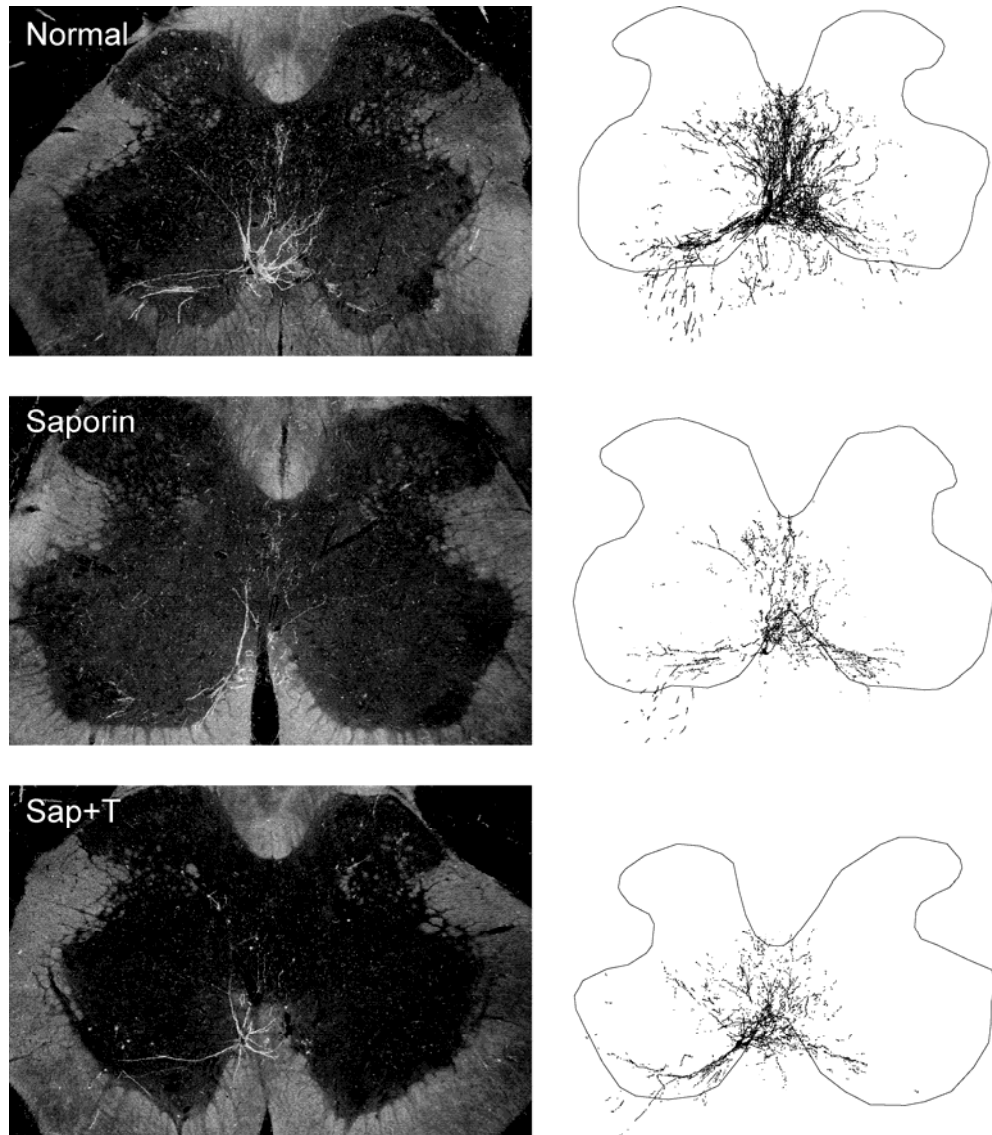


FIGURE 3.5

Left: Darkfield photomicrographs of transverse sections through the lumbar spinal cords of a normal control, an untreated saporin-injected animal, and a saporin-injected animal treated with testosterone, after HRP injection into the left LA muscle. Right: Computer-generated composites of HRP-labeled somata and processes drawn at 320  $\mu\text{m}$  intervals through the entire rostrocaudal extent of the SNB; these composites were selected because they are representative of their respective group average dendritic lengths.

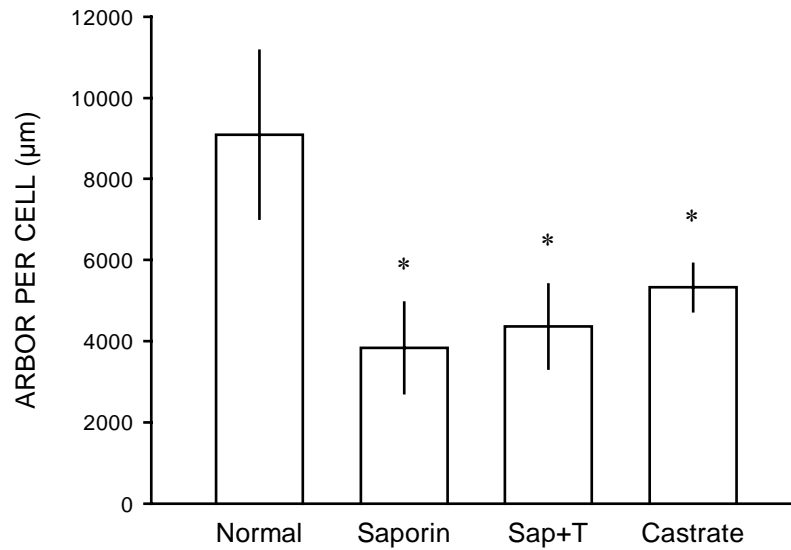


FIGURE 3.6

Dendritic lengths of LA-projecting SNB motoneurons for normal controls, untreated saporin-injected animals, saporin-injected animals treated with testosterone, and castrated controls. Saporin injection into the BC muscle reduced the dendritic length of ipsilateral LA-projecting motoneurons by almost 60%. Treatment with testosterone completely failed to prevent this atrophy. \* indicates significantly different from normal controls,  $p < .05$ . Bar heights represent means  $\pm$  SEM.

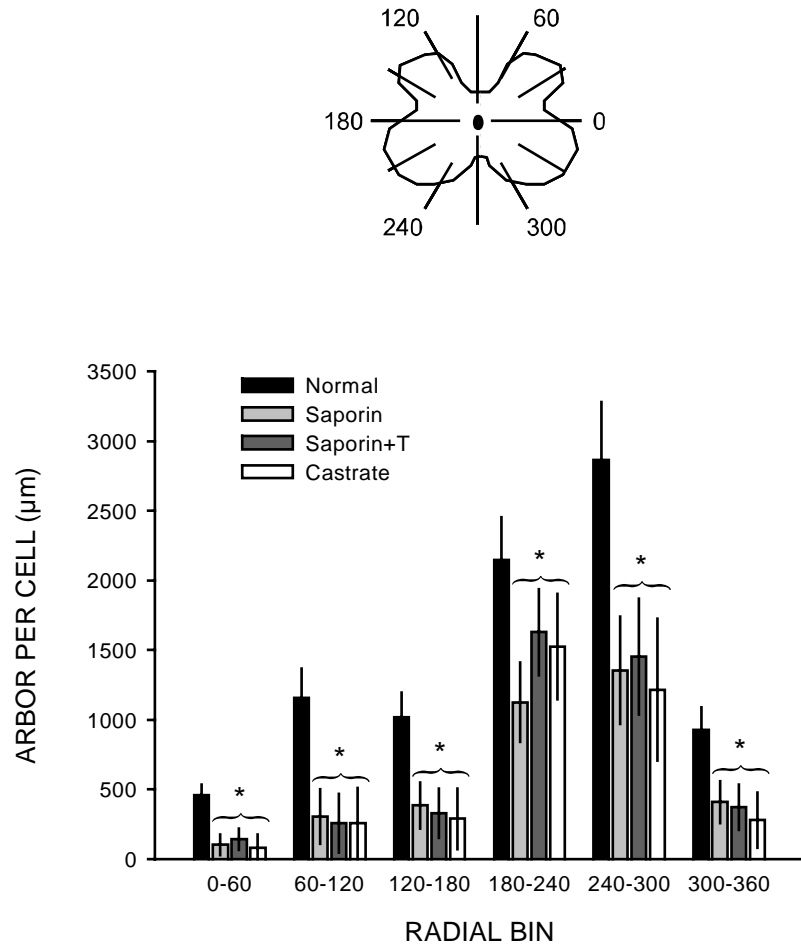


FIGURE 3.7

Top: Drawing of spinal gray matter divided into radial sectors for measure of SNB dendritic distribution. Bottom: Length per radial bin of SNB dendrites in normal controls, untreated saporin-injected animals, saporin-injected animals treated with testosterone, and castrated controls. For graphic purposes, dendritic length measures have been collapsed into 6 bins of 60° each. Saporin injection reduced dendritic length in every bin, and testosterone treatment did not prevent this. \* indicates significantly different from normal controls,  $p < .05$ . Bar heights represent means  $\pm$  SEM.

Radial dendritic extent was nonuniform across bins, and repeated-measures ANOVA revealed a significant effect of radial location [ $F(11, 209) = 67.96, p < .001$ ]. However, radial dendritic extent was not affected by saporin injection, testosterone treatment, or castration: it did not differ between groups [ $F(3, 19) = 2.40, ns$ ], and there was no interaction between radial location and group [ $F(33, 209) = 1.02, ns$ ; see Figure 3.8]. Additionally, rostrocaudal dendritic extent for normal controls ( $3226.67 \pm 275.33 \mu\text{m}$ ), saporin-injected animals ( $2742.86 \pm 176.06 \mu\text{m}$ ), saporin-injected animals treated with testosterone ( $2666.67 \pm 173.64 \mu\text{m}$ ), and castrated controls ( $3040.00 \pm 146.06 \mu\text{m}$ ) did not differ each other [ $F(3, 19) = 1.63, ns$ ; grand mean  $2900.87 \pm 108.48 \mu\text{m}$ ].

## DISCUSSION

### Muscle Weights

Saporin injection into the BC muscle decreased BC muscle weight (Figure 3.1, left). This is completely consistent with the previous experiments (Fargo and Sengelaub, 2004a, 2004b) and with the experiments described in Chapters 4 and 6 of this thesis, in which injection of saporin into both the BC and LA resulted in dramatic weight loss in the injected musculature.

Testosterone treatment completely prevented the saporin-induced atrophy of the BC muscle (Figure 3.1, left). This serves as an important manipulation check in this experiment, given that testosterone treatment failed to prevent atrophy of the LA-projecting motoneurons. Testosterone treatment does not always prevent the muscular atrophy induced by saporin injection. In one previous experiment (Fargo and Sengelaub, 2004a) testosterone treatment prevented atrophy of the saporin-injected muscles, while in another (Fargo and Sengelaub, 2004b) it did not. In the experiment described in Chapter



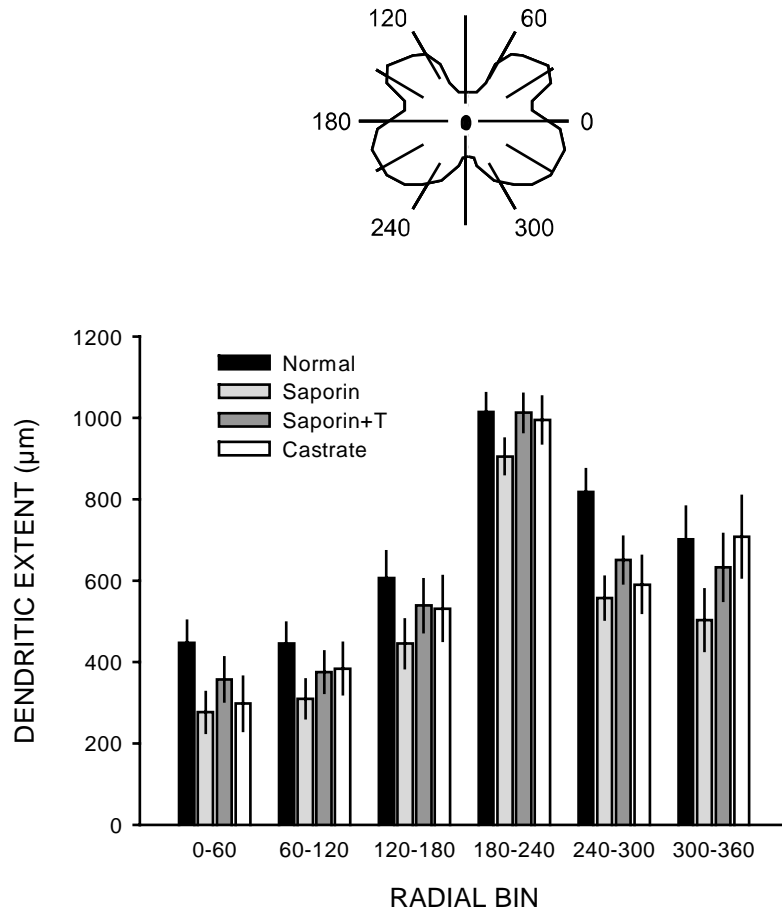


FIGURE 3.8

Top: Drawing of spinal gray matter divided into radial sectors for measure of SNB radial dendritic extent. Bottom: Radial extents of SNB dendrites in normal controls, untreated saporin-injected animals, saporin-injected animals treated with testosterone, and castrated controls. For graphic purposes, dendritic extent measures have been collapsed into 6 bins of 60° each. SNB radial dendritic extent is non-uniform across radial bins. However, radial dendritic extent did not differ between groups, nor was there any interaction between group and radial bin. Bar heights represent means  $\pm$  SEM.

6 of this thesis testosterone treatment attenuated, but did not completely prevent, atrophy of the saporin-injected musculature.

In the current experiment, the motoneurons labeled with HRP and visualized for morphometry were LA-projecting SNB motoneurons ipsilateral to the saporin injection. The weight of the ipsilateral LA was not significantly affected by either saporin injection or treatment with exogenous testosterone (Figure 3.1, right). The fact that LA weight was not affected by testosterone treatment was unexpected. In previous experiments (Fargo and Sengelaub, 2004a, 2004b) and in the experiment described in Chapter 6, testosterone treatment has led to the hypertrophy of the perineal musculature not injected with saporin. Indeed, testosterone is a powerful anabolic steroid, and the weight of the perineal musculature is particularly responsive to its manipulation (Eisenberg et al., 1949; Wainman and Shipounoff, 1941).<sup>1</sup> Perhaps the saporin injected into the nearby BC muscle leaked in the periphery and spread to the LA. If saporin did indeed leak and spread to the LA, there was not enough of it to cause LA atrophy, but there may have been enough to attenuate testosterone's ability to cause muscular hypertrophy in the LA.

The fact that the LA did not suffer atrophy in the untreated saporin-injected animals is an important observation. I argue that the dendritic atrophy observed in LA-projecting motoneurons following saporin injection into the BC was secondary to the death of BC-projecting motoneurons. As mentioned above, though, it is possible that

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<sup>1</sup> It is interesting to note that testosterone was ineffective both in causing LA hypertrophy and in rescuing LA-projecting motoneurons from dendritic atrophy following saporin injection into the BC. It is even possible that the two results are causally linked. It is known that denervation abolishes the ability of testosterone treatment to increase LA muscle weight and protein synthesis (Burešová et al., 1972; Rand and Breedlove, 1992). Perhaps the atrophy observed in LA-projecting motoneurons is so profound that they are rendered incapable of activating the LA. Such a functional denervation of the LA could be expected to abolish the ability of testosterone to cause muscular hypertrophy. Of course, this might also be expected to lead to the kind of atrophy observed after mechanical denervation of the perineal musculature (Burešová et al., 1972; Chapter 5 of this thesis), which it did not do.

saporin leaked from the BC and spread to the LA in the periphery, where it could then have acted *directly* on LA-projecting motoneurons. This alternative explanation for the effect of saporin injection on the dendritic morphology of LA-projecting motoneurons cannot be ruled out by the current data. However, the fact that the LA's weight was unchanged in untreated saporin-injected animals increases my confidence that saporin did not act directly at the LA. This observation is in concert with previous experiments (Fargo and Sengelaub, 2004a, 2004b) and with the experiment described in Chapter 6 of this thesis, in which saporin injection into one side of the BC/LA did not alter muscle weights on the uninjected side.

### Cell Counts

Injection of saporin into the BC muscle resulted in the death of over 40% of the motoneurons in the ipsilateral SNB (Figure 3.2). Although it is not possible to determine whether this cell death was specific to BC-projecting motoneurons, there are at least three reasons to make this assumption. First, in previous experiments (Fargo and Sengelaub, 2004a, 2004b) and in the experiment described in Chapter 6, saporin injection into one side of the BC/LA musculature did not result in any motoneuron death on the other side of the animal, and the BC and LA muscles on either side of the body are just as proximal to each other as are the BC and LA muscle on one side. Second, the number of motoneurons labeled with HRP in this experiment did not differ between groups. Recall that the HRP-labeled motoneurons in this experiment project to the LA; if LA-projecting motoneurons were killed by injection of saporin into the BC muscle, then I would expect fewer of them to be labeled in the saporin-injected groups. Third, injection of saporin into the BC only resulted in the death of around 40% of ipsilateral SNB motoneurons, while

injection of saporin into both the BC *and* the LA results in the death of more than 60% of ipsilateral SNB motoneurons (Fargo and Sengelaub, 2004a, 2004b; Chapter 6); presumably, the additional 20% or so are LA-projecting motoneurons. For these reasons, I am making the assumption that saporin injection into the BC in this experiment resulted in the death of only BC-projecting motoneurons.

In some experimental paradigms, treatment with exogenous steroids prevents injury-induced motoneuron death ( e.g., Ahlbom et al., 2001; Hammond et al., 2001; Huppenbauer et al., 2005; Pike, 2001; Ramsden et al., 2003). It is therefore important to note that saporin injection resulted in motoneuron death regardless of testosterone treatment in the present experiment. Because of this, any potential beneficial effect of testosterone treatment on the morphology of the remaining, LA-projecting motoneurons cannot be attributed to testosterone attenuating the motoneuron depletion induced by saporin injection.

### Morphometry

The number of motoneurons labeled with HRP did not differ between groups. Therefore, it is unlikely that differences in measures of motoneuron morphology between groups were affected by potential differences in the number of motoneurons labeled with HRP.

### Somata

Group differences in mean cross-sectional soma area did not reach significance (Figure 3.3). Despite this, castrated controls had smaller somata than any other group, and castration has long been known to reliably shrink SNB somata (Araki et al., 1991; Breedlove and Arnold, 1981; Collins et al., 1992; Fargo and Sengelaub, 2004a; Forger et

al., 1992). The most important point to make here, though, is that saporin injection into the BC muscle did not result in somal atrophy in ipsilateral LA-projecting motoneurons. This result stands in contrast with earlier studies (Fargo and Sengelaub, 2004a, 2004b) and the experiment described in Chapter 6, in which saporin injection into BC and LA resulted in the somal atrophy of contralateral BC-projecting motoneurons. While this might be attributed to a simple lack of sensitivity in the current study, it should be noted that the technique I used to measure soma area in this study is no different from what I used in the other studies. One might still argue that this technique is less sensitive for LA-projecting motoneurons than for BC-projecting motoneurons, but the large difference between castrates and the other groups makes this explanation unlikely. Given the lack of a plausible alternate explanation, I must conclude that there really was no effect of saporin injection into the BC muscle on soma areas of ipsilateral LA-projecting motoneurons.

It is difficult to interpret this difference between studies because the measured cells in the current study differ from the others in at least three important ways—first, they are LA-projecting rather than BC-projecting motoneurons; second, they are ipsilateral rather than contralateral to the saporin injection; and third, only one rather than two of their neighboring subpopulations has been depleted by saporin injection. The lack of an effect of saporin injection might be attributable to any of these differences.

Potential group differences in soma areas were also assessed by creating frequency distributions of the individual soma areas in each group. The results of this analysis were similar to those obtained by examining mean soma areas. Neither saporin injected animals nor saporin-injected animals treated with testosterone had frequency

distributions that differed from that of normal controls. Only castration alone changed the distribution of individual soma areas. Castrated controls had more small somata and fewer medium and large somata.

### Dendrites

Injection of saporin into the BC muscle resulted in significant dendritic atrophy of ipsilateral LA-projecting motoneurons (Figure 3.6). This result is in line with previous experiments (Fargo and Sengelaub, 2004a, 2004b), and along with the results of the experiment described in Chapter 6, provides important confirmatory evidence that motoneuron depletion by intramuscular saporin injection leads to atrophy of surviving motoneurons in nearby pools. Indeed, even the amount of dendritic retraction (approximately 60%) was very similar to what is seen in other studies.

Surprisingly, however, treatment with exogenous testosterone had absolutely no beneficial effect on dendritic length. As with soma area, this result stands in stark contrast with previous studies (Fargo and Sengelaub, 2004a, 2004b) and the results of the experiment described in Chapter 6. Unfortunately, it is equally as difficult to interpret, primarily because the mechanism of action for testosterone in this model is unknown. For example, if testosterone exerts its protective effects on BC-projecting motoneurons by acting directly on the neurons, then one might speculate that the lack of a protective effect of testosterone treatment in the current experiment is due to some intrinsic characteristic of LA-projecting motoneurons that makes them less sensitive to testosterone than BC-projecting motoneurons.

Alternatively, if the site of action for testosterone is at the muscle, one might speculate that the LA is not capable of responding to testosterone treatment as vigorously

as does the BC. In this case, it might be that some trophic factor that protects motoneurons is upregulated by the BC, but not the LA, in response to testosterone treatment. In fact, this is an attractive hypothesis. In the present experiment testosterone treatment had no effect on LA muscle weight (Figure 3.1), but in previous studies (Fargo and Sengelaub, 2004a, 2004b) and in the experiment described in Chapter 6, in which testosterone treatment was protective of dendritic length, the measured motoneurons projected to muscles that were significantly hypertrophied in the testosterone-treated animals. So there is at least a correlation between muscle hypertrophy and dendritic protection in testosterone-treated animals.<sup>2</sup> Moreover, it is known that testosterone depends on the presence of BDNF, probably supplied by the muscle *in vivo*, to support normal dendritic length in the SNB (Yang et al., 2004).

Of course, other possible explanations exist for the lack of a protective effect of testosterone on LA-projecting motoneurons in this experiment. For example, connectivity between the affected subpopulations of motoneurons might be different between this experiment and previous experiments. BC-projecting motoneurons are connected to contralateral SNB motoneurons by gap junctions (Coleman and Sengelaub, 2002), and BC-projecting motoneurons are protected from dendritic atrophy when the contralateral SNB motoneurons are killed by intramuscular saporin injection (Fargo and Sengelaub, 2004a, 2004b; Chapter 6). In contrast, it has never been shown that LA-projecting motoneurons are connected by gap junctions to other ipsilateral SNB motoneurons—and LA-projected motoneurons are *not* protected when ipsilateral BC-projecting motoneurons are killed. It is therefore at least reasonable to wonder if testosterone's inability to protect

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<sup>2</sup> However, the results reported in Chapter 6, with special attention paid to the dihydrotestosterone-treated group, suggest that muscle hypertrophy is not a necessary precondition for androgenic protection of SNB dendritic length.

LA-projecting motoneurons in this experiment is related to a potential lack of connectivity between those motoneurons and the BC-projecting motoneurons that were killed. A similar hypothesis arises from the fact that LA-projecting motoneurons are spatially intermingled with ipsilateral BC-projecting motoneurons while the somata in the contralateral motoneuron pools are completely separated (McKenna and Nadelhaft, 1986). Perhaps testosterone is incapable of rescuing motoneuron dendrites from atrophy when the somata of the measured neurons are closely intermingled with those that are killed.<sup>3</sup>

The possibility that saporin injection could depress retrograde transport of HRP and produce spuriously low measures of dendritic length is an important consideration. However, measures of dendritic extent would also be affected by depression of retrograde transport, and in the current experiment neither radial dendritic extent (Figure 3.8) nor rostrocaudal dendritic extent differed between groups, making this unlikely.

### Conclusions

The purpose of the present experiment was to examine whether or not testosterone treatment would protect motoneurons from regressive changes following the death of ipsilateral motoneurons. Dendritic length was markedly reduced in saporin-injected animals regardless of treatment with testosterone, suggesting that testosterone treatment is *not* capable of producing such a protective effect. However, these results are made difficult to interpret further because the motoneurons examined in this experiment differ from those in previous experiments in multiple ways, including their spatial relationship

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<sup>3</sup> However, preliminary data from our laboratory suggest that, following saporin-induced loss of quadriceps motoneurons, testosterone treatment rescues intermingled surviving ipsilateral quadriceps motoneurons from dendritic atrophy. Note that this also argues against the possibility that the protective effect of testosterone is “contralateral only”.



to the depleted motoneurons, their target musculature (including its lack of hypertrophy in response to testosterone treatment), and the fact that their somata did not shrink following saporin injection.

Also, based solely on the data from this experiment, one cannot rule out the possibility that saporin simply leaked from the BC and spread to the LA in the periphery, where it acted directly on the LA muscle and LA-projecting motoneurons. This would explain the lack of a testosterone effect in both LA muscle weight and LA-projecting motoneuron dendritic morphology. Recently, our laboratory has begun injecting saporin into the thigh muscle, *vastus medialis*, then examining the morphology of the ipsilateral *vastus lateralis*, another thigh muscle. This experimental setup is similar to the present experiment in that the depleted motoneurons and the motoneurons assessed for morphometry are intermingled within the same motor pool, and different from the present experiment in that the two muscles are located far enough away from each other as to make peripheral spread of saporin to the uninjected muscle highly unlikely. Preliminary unpublished observations indicate that testosterone treatment is effective in preventing saporin-induced motoneuron atrophy in that experimental design, further supporting the idea that the lack of a testosterone effect in the present experiment was due to leakage and peripheral spread of saporin. Future experiments must address this issue. The simplest way to rule out the leakage explanation would be to institute experimental controls that would prevent leakage of saporin from the BC muscle, for instance by closing the needle hole in the muscle with a drop of cyanoacrylate. Alternatively, protein expression could be examined in the BC, LA, and control muscles to rule out a direct effect of saporin on the LA in the periphery.

## Chapter 4.

Does Testosterone Protect against Deficits in Motor Activation Caused by Depletion of  
Contralateral Motoneurons?

Motoneuron loss is a prominent feature of several movement disorders as well as a consequence of traumatic injuries of the spinal cord. However, motor systems have a high degree of redundancy, and display remarkable plasticity in the face of various insults. It has been suggested that this redundancy and plasticity may serve as a substrate for treatment strategies in disease or after spinal cord lesions (Edgerton et al., 2004). Given that we are, as yet, incapable of replacing lost motoneurons, the best hope for retaining normal function following motoneuron loss may lie in promoting the health of surviving motoneurons. My previous experiments (Fargo and Sengelaub, 2004a, 2004b) demonstrated a profound effect of testosterone in rescuing motoneurons from somal and dendritic atrophy following the death of their contralateral counterparts. However, while the effects of testosterone treatment on the morphology of the surviving motoneurons are striking, it is unknown whether they confer any functional benefit to the animal. The purpose of the present experiment, therefore, was to examine the electrophysiological response properties of SNB motoneurons following saporin injection, and to determine whether treatment with exogenous testosterone would prevent any possible electrophysiological deficits caused by saporin injection.

In males, the motoneurons of the SNB innervate the perineal BC and LA muscles, as well as the external anal sphincter (Breedlove and Arnold, 1980; McKenna and Nadelhaft, 1986; Schröder, 1980). These BC and LA surround the base of the penis, and their coordinated activity mediates penile reflexes. Specifically, they produce strong, rhythmic contractions resulting in a cupping or flaring of the glans penis (Hart and Melese-D'Hospital, 1983). Penile cupping is necessary for depositing seminal plugs in the vaginal canal, and for removing those deposited previously by a competing male, both

critically important for successful copulation (Sachs, 1982). Accordingly, during intromission and ejaculation, the BC muscle contracts with very high frequency and amplitude (Holmes et al., 1991), and the activity of the system can be thought of as producing an “all or nothing” response.

Several aspects of the spinal circuitry of the SNB system have been established. While many spinal reflex pathways are monosynaptic, multiple lines of evidence indicate that the SNB reflex pathway is polysynaptic. The pudendal nerve afferents terminate in the dorsal gray commissure (McKenna and Nadelhaft, 1986), the site of a large population of interneurons that terminate on SNB motoneurons (Collins et al., 1991). In addition, the relatively long delay between stimulation of the pudendal afferents and the onset of activity in SNB motor axons (~4.5 msec) is inconsistent with a monosynaptic circuit, even at the highest recorded conduction velocities of SNB afferent and efferent fibers (McKenna and Nadelhaft, 1989). Furthermore, stimulation of the pudendal afferents produces a mixture of both IPSPs and EPSPs in SNB motoneurons (Collins, 1985).

Another prominent feature of the SNB spinal circuitry is its bilateral organization. Afferent pudendal nerve axons arborize in both the ipsilateral and contralateral dorsal gray commissure (McKenna and Nadelhaft, 1986). Additionally, the dendritic fields of the left and right sides of the SNB overlap extensively, and SNB motoneuron dendrites even directly appose the somata of contralateral SNB motoneurons (Kurz et al., 1986; Rose and Collins, 1985). Moreover, SNB motoneurons are connected to their contralateral counterparts via gap junctions (Coleman and Sengelaub, 2002). The bilateral organization of the SNB spinal circuitry gives rise to bilateral patterns of activity in the

penile reflexes, with stimulation of the pudendal afferents producing activity in both ipsilateral and contralateral SNB motor axons (Foster and Sengelaub, 2004a; McKenna and Nadelhaft, 1989; Tanaka and Arnold, 1993).

As reviewed in Chapter 1, several features of SNB spinal circuitry are dependent on steroid hormones. Among these, of particular importance in SNB motoneurons are the morphology of both somata and dendrites (Breedlove and Arnold, 1981; Kurz et al., 1986; Sasaki and Arnold, 1991), the amount of synaptic input (Leedy et al., 1987; Matsumoto et al., 1988), the number and size of gap junction plaques (Matsumoto et al., 1988), and the functional expression of N-type  $Ca^{2+}$  channels at the motor end plates (Nudler et al., 2005). Accordingly, steroid hormones are necessary to maintain normal electrophysiological responses in this reflex pathway. Removal of endogenous gonadal steroids by castration decreases the number of cups produced reflexively in response to retraction of the preputial sheath, and testosterone replacement reverses this (Hart, 1967). In addition, castration increases response latency, attenuates SNB motoneuron recruitment, and decreases the number of EMG spikes produced in response to afferent stimulation, while treatment with exogenous gonadal steroids prevents all of these deficits (Fargo et al., 2003; Foster and Sengelaub, 2004b; Holmes and Sachs, 1992). These facts suggest that the protective effects of testosterone on SNB morphology following saporin injection may attenuate any possible deficits in motor activation caused by saporin injection.

While the electrophysiology of injured motoneurons has been a subject of some study, there are only a few reports in the literature concerning the electrophysiology of surviving motoneurons in close proximity to an injury site. What studies there are have

been conducted by J.-O. Kellerth and his colleagues, working in cat. Their most recent studies (Havton et al., 2001; Havton and Kellerth, 2004) have utilized nerve injuries that do not result in motoneuron death, and shed very little light on the current dissertation work. However, in their original experimental setup, they killed lumbosacral motoneurons unilaterally by avulsion of the L7 ventral root (cats have 7 lumbar spinal segments, whereas rats have only 6). The lumbosacral dorsal roots were then stimulated bilaterally, and recordings were taken from the remaining lumbosacral ventral roots bilaterally. The authors reported a two-fold difference in the amplitude of reflex responses between the contralateral side and the ipsilateral side, with the ipsilateral side displaying larger responses (Holmberg and Kellerth, 1996; Holmberg and Kellerth, 2000). The authors interpreted this as an increase in reflex strength on the ipsilateral side, but because only internal controls were used, it is also possible that what they saw was actually a decrease in strength of the reflex pathway on the side of the animal contralateral to the lesion.

The present experiment was designed to determine what electrophysiological changes might accompany the motoneuron atrophy induced by contralateral saporin injection, and moreover, to determine whether treatment with exogenous testosterone would prevent these changes as it prevents motoneuron atrophy. To accomplish this goal, I studied the electrophysiological response properties of SNB motoneurons contralateral to saporin injection, both with and without testosterone treatment.

## METHODS AND DESIGN

### Animals

Adult male Sprague Dawley rats were used. Saporin injections were made into the BC and LA muscles according to the method described in Chapter 2. Coincident with saporin injection, some animals were also simultaneously castrated and given replacement testosterone according the methods described in Chapter 2 (saporin only  $n = 6$ , saporin and testosterone treatment  $n = 5$ ). A group of untreated intact normal males ( $n = 5$ ) and a group of untreated castrates ( $n = 6$ ) served as control groups.

### Peripheral Nerve Recording

Four weeks after saporin injection and testosterone-treatment onset, I proceeded with peripheral nerve recording as described by Foster and Sengelaub (2004a). Peripheral nerve recording is a somewhat misleading name for the procedure, as it is designed specifically to isolate and record from the central components of the neuromuscular circuit (proximal nerves, central synapses, interneurons, and motoneurons), and not the peripheral components (distal nerves, dorsal root ganglia, neuromuscular junctions, and muscles). For a schematic representation of peripheral nerve recording as used in this experiment, see Figure 4.1.

Each animal was weighed then placed under general anesthesia with an intraperitoneal injection of chloral hydrate (450 mg/kg body weight, with supplemental doses as needed to maintain areflexia to painful stimuli). Once the animal no longer responded to pain, it was placed in the prostrate position on a 37 °C heating pad, and an incision of approximately 2 cm was made through the skin, parallel to the spine and above the second thoracic vertebra. The skin was retracted with ophthalmic spreaders,

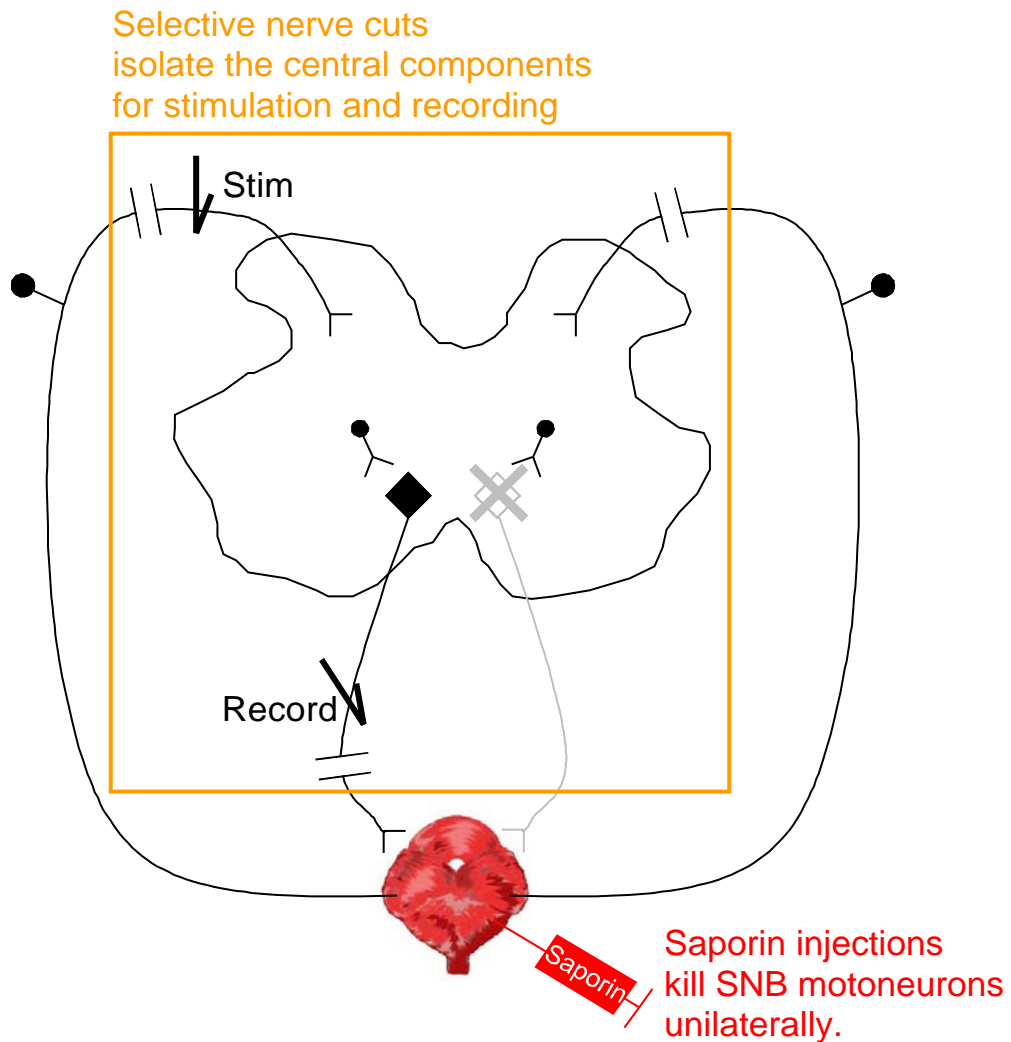


FIGURE 4.1

Schematic representation of the procedures used in this experiment. Projections are drawn to illustrate the locations of nerve cuts and electrode placement, and are not intended to be representative of the anatomical details of the spinal circuitry.



and a scalpel was used to cut through the paraspinous muscles and connective tissue until the dorsal aspects of several thoracic vertebrae were completely exposed. The muscles and connective tissue were retracted with ophthalmic spreaders, and a few minutes were allowed to pass so that bleeding would subside before exposing the central nervous tissue. I then performed a dorsal laminectomy of the thoracic vertebra with the most prominent spinous process, and completely transected the spinal cord directly under this process. Spinal cord transection removes descending tonic inhibition of spinal reflexes, allowing for a more accurate characterization of their properties. The exposed tissue was covered with mineral oil to prevent desiccation, and the skin was closed with 9-mm AutoClips brand stainless steel wound clips.

Another incision was made through the skin parallel to the spine, approximately 6 cm in length, and placed above the thirteenth thoracic and the first and second lumbar vertebrae. A scalpel was used to cut through the paraspinous muscles and connective tissue until the dorsal aspects of the vertebrae were completely exposed. The soft tissue was then carefully removed from between the transverse and articulating processes, allowing the fitting of a custom-made spinal clamp used to stabilize the spine and reduce potential movement artifacts during recording. After affixing the spinal clamp, I performed a dorsal laminectomy of the thirteenth thoracic vertebra and the first and second lumbar vertebrae, and carefully removed the dura from the underlying tissue. This exposed the caudal portion of the lumbar enlargement, in which the motoneurons of the SNB reside, and allowed for the visualization of the dorsal roots L5-S1. The dorsal root L6 contralateral to saporin injection was draped over a bipolar stimulating electrode and crushed to it by ligation with a short length of surgical silk suture. (The contralateral

dorsal root was chosen for stimulation because of the possibility that saporin injection resulted in damage to the ipsilateral dorsal root.) Dorsal roots L5-S1 were then severed (distal to the electrode) on both sides of the animal in order to reduce unwanted stimulation of the SNB by motor afferents. The exposed spinal region was then bathed in mineral oil to prevent desiccation, and additional applications of mineral oil were administered periodically throughout recording.

The tail was then elevated and immobilized, and an incision was made between the tail and the anus. The perineal muscles and the nerves that supply them were exposed by blunt dissection. The pudendal nerve motor branch carrying the efferents from the SNB to the BC (contralateral to saporin injection) was draped over a bipolar hook wire recording electrode (model PBCA0750; FHC, Bowdoinham, ME) and crushed to it by ligation with a short length of surgical silk suture. The nerve was then severed distal to electrode placement to prevent any possible trans-segmental feedback from the periphery. The exposed region was then bathed in mineral oil to prevent desiccation, and additional applications of mineral oil were administered periodically throughout recording. A ground wire was attached to the animal's tail. Care was taken to attach both the stimulating and recording electrodes in the same locations in each animal.

A computer-based stimulation and recording system (SuperScope II; GWI, Somerville, MA) was used to drive an S48 stimulator (Grass, West Warwick, RI). The stimulus pulse was passed through a constant current unit (model PSIU6E; Grass) and a bipolar hook wire stimulating electrode (model PBCA0750; FHC). In order to record the actual current delivered during each stimulation, a current probe was attached between the constant current unit and the stimulating electrode. The signal from the recording

electrode was passed through an A-M Systems (Carlsborg, WA) differential AC amplifier (Model 1700), filtered (low: 300 Hz; high: 20 kHz), and amplified 1000X. Signals from both the current probe and the recording electrode were sent to an analog-to-digital acquisition device (InstruNet Model 100B; GWI, Somerville, MA) and recorded by SuperScope II at an acquisition rate of 10 kHz. Background activity of the motor nerve was recorded for approximately 13 msec before each stimulus pulse. Stimulus pulses 0.25 ms long were generated once every 15 seconds, and resultant motor nerve activity was recorded for approximately 87 ms after each stimulus onset. In order to prevent polarization of the stimulating electrodes, current was temporarily reversed after every 33 or 34 stimulus pulses (with recording suspended). For each animal, the threshold stimulus intensity and the stimulus intensity generating the maximum response were determined empirically. Stimulus intensity was then varied in order to sample from the entire range of effective stimulus intensities. Approximately 200 traces were generated for each animal. Following the completion of recording, animals were killed by an overdose of urethane and the BC and LA muscles were removed and weighed.

#### Data Analysis

Response latency was determined by measuring the delay between the onset of the stimulus pulse at the dorsal root and the beginning of the first spike (defined as at least 10 times the rectified amplitude of the average background activity) in the motor nerve. Activity length was measured by determining the absolute amount of time between the beginning of the first spike and the end of the last spike following a stimulus pulse. In addition, the number of spikes generated following each stimulus pulse was counted.

The maximum response of each animal was measured in two ways. First, the maximum peak-to-peak amplitude was determined in the unrectified data. Second, the traces were rectified and the maximum value for area under the curve was identified. Next, recruitment curves were constructed. Recruitment curves plot response magnitude as a function of stimulus intensity. Due to individual differences and minor changes in electrode placement, the range of stimulus intensities that produced a motor nerve response varied between animals. To minimize the effects of this variability, stimulus intensities were standardized across animals. This was accomplished by identifying for each animal the lowest stimulus intensity that produced a maximum response (the “lowest maximally effective stimulus”), then expressing all other stimulus intensities for that animal as a percentage of that stimulus intensity. Recruitment curves were constructed with the unrectified data using peak-to-peak amplitude as the measure of response magnitude, and with the rectified data using area under the curve as the measure of response magnitude. Because the maximum peak-to-peak response and the maximum area under the curve response for each animal were not always produced by the same stimulus pulse, the stimulus intensities were standardized separately in these two analyses.

To minimize the possibility of comparisons being affected by differences in motor unit recruitment, all measures were taken at high levels of stimulus intensity (except for response magnitudes measured during the construction of recruitment curves, which are by definition measured over the entire range of response-producing stimuli). Thus, response latencies were measured only for traces resulting from stimulus intensities at or above 90% of the lowest maximally effective stimulus (as determined by peak-to-peak

amplitude) for each animal. Similarly, average background activity, activity duration, and spike number were measured only for traces resulting from stimulus intensities between 90% and 110% of the lowest maximally effective stimulus (as determined by peak-to-peak amplitude) for each animal.

All results were analyzed by analyses of variance (one-way or repeated measures as appropriate) followed by post hoc analyses using Fisher's least significant difference (LSD).

## RESULTS

### Muscle Weights

BC and LA muscle weights were recorded only for the saporin-injected groups. Unilateral saporin injection into the BC and LA muscles resulted in significant atrophy of the BC/LA on the injected side [compared to the non-injected side as an internal control;  $F(1, 8) = 48.17, p < .001$ ; see Figure 4.2]. However, testosterone treatment increased BC/LA muscle weight on both the non-injected [ $0.672 \pm 0.009$  g for saporin-injected animals treated with testosterone compared to  $0.575 \pm 0.018$  g for untreated saporin-injected animals;  $F(1, 7) = 25.62, p < .01$ ] and saporin-injected [ $0.527 \pm 0.009$  g for saporin-injected animals treated with testosterone compared to  $0.423 \pm 0.035$  g for untreated saporin-injected animals;  $F(1, 7) = 10.25, p < .05$ ] sides.

### Background Activity

Average background activity differed significantly by group [ $F(3, 16) = 7.68, p < .01$ ; see Figure 4.3]. An examination of the individual data points reveals an outlier (see Figure 4.4). Of the five animals in the untreated saporin-injected group for whom background activity was recorded, one had a higher value than any other animal in any

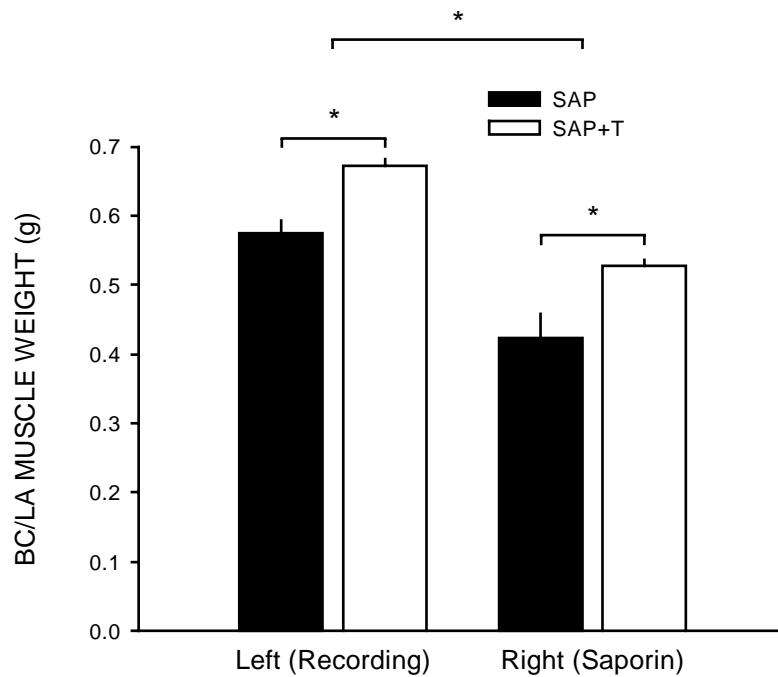


FIGURE 4.2

BC/LA muscle weights for untreated saporin-injected animals (filled bars) and saporin-injected animals treated with testosterone (open bars), for both the recording (left) and saporin-injected (right) sides. Saporin injection decreased BC/LA weight, and treatment with exogenous testosterone increased BC/LA muscle weight. \* indicates significant difference,  $p < .05$ . Bar heights represent means  $\pm$  SEM.

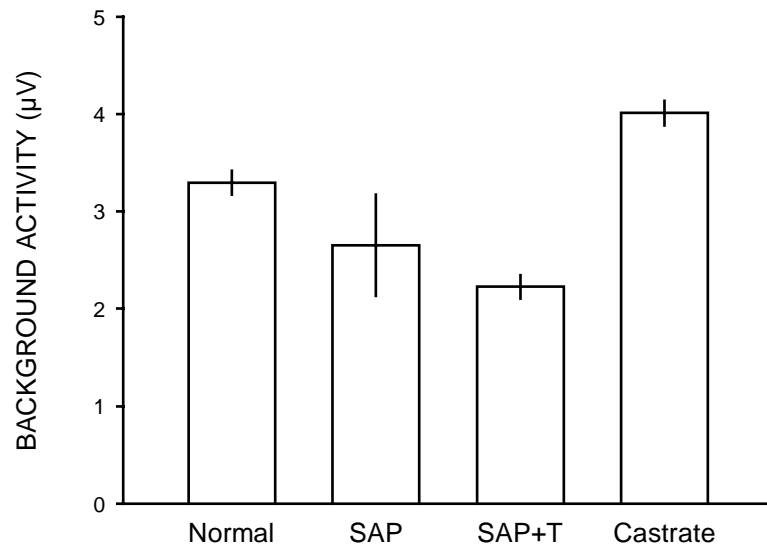


FIGURE 4.3

Average background activity for normal controls, untreated saporin-injected animals, saporin-injected animals treated with testosterone, and castrated controls. Average background activity varied significantly with group. Note that the variance of the untreated saporin-injected group is much larger than for any other group. Bar heights represent means  $\pm$  SEM.

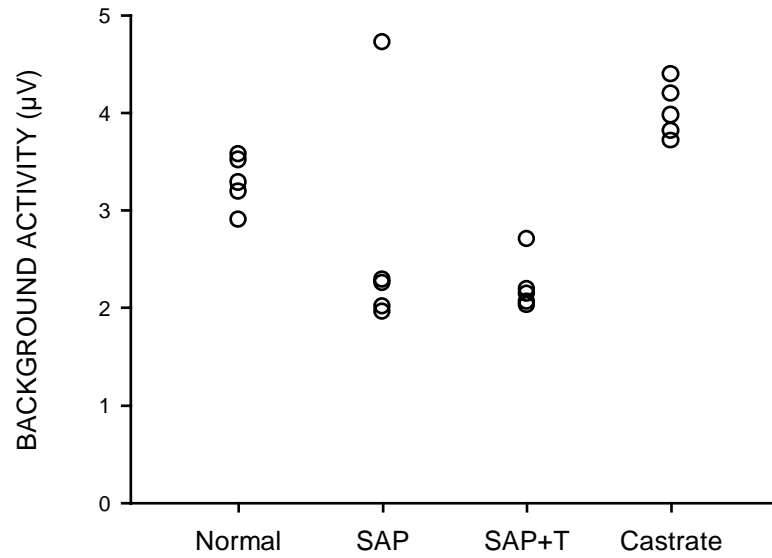


FIGURE 4.4

Average background activity of individual normal controls, untreated saporin-injected animals, saporin-injected animals treated with testosterone, and castrated controls. Note the outlier in the untreated saporin-injected group that was higher than any other data point in any group.



group ( $4.72 \mu\text{V}$ ). Removing that data point clarifies the effects of saporin and testosterone on average background activity (see Figure 4.5). Saporin injection significantly reduced average background activity ( $2.13 \pm 0.07 \mu\text{V}$  for saporin-injected animals compared to  $3.30 \pm 0.12 \mu\text{V}$  for normal controls; LSD,  $p < .001$ ), and treatment with testosterone did not prevent this decrease ( $2.23 \pm 0.12 \mu\text{V}$  for saporin-injected animals treated with testosterone, compared to normal controls; LSD,  $p < .001$ ). Castration significantly increased average background activity ( $4.01 \pm 0.13 \mu\text{V}$  for castrated controls, compared to normal controls; LSD,  $p < .001$ ).

### Evoked Activity

In normal controls, activity produced by high intensity stimuli began  $2.74 \pm 0.34$  msec after delivery of the stimulus pulse, on average. However, response latency was significantly lengthened by saporin injection [ $4.93 \pm 0.50$  msec for saporin-injected animals; compared to normal controls, LSD,  $p < .001$ ; overall test for the effect of group on response latency  $F(3, 16) = 12.58$ ,  $p < .001$ ; see Figure 4.6]. Treatment with testosterone did not attenuate saporin-induced increases in response latency ( $4.95 \pm 0.43$  msec for saporin-injected animals treated with testosterone; compared to normal controls, LSD,  $p < .001$ ). Castration had no effect on response latency ( $2.46 \pm 0.21$  msec for castrated controls; compared to normal controls, LSD, *ns*).

There were also differences in the duration of activity in response to high intensity stimulation [ $F(3, 16) = 3.60$ ,  $p < .05$ ; see Figure 4.7]. In normal controls, activity lasted for an average of  $11.66 \pm 1.30$  msec, and never persisted for longer than 17 msec. Saporin injection greatly increased activity duration ( $30.09 \pm 6.97$  msec for saporin-injected animals; compared to normal controls, LSD,  $p < .01$ ), and only one

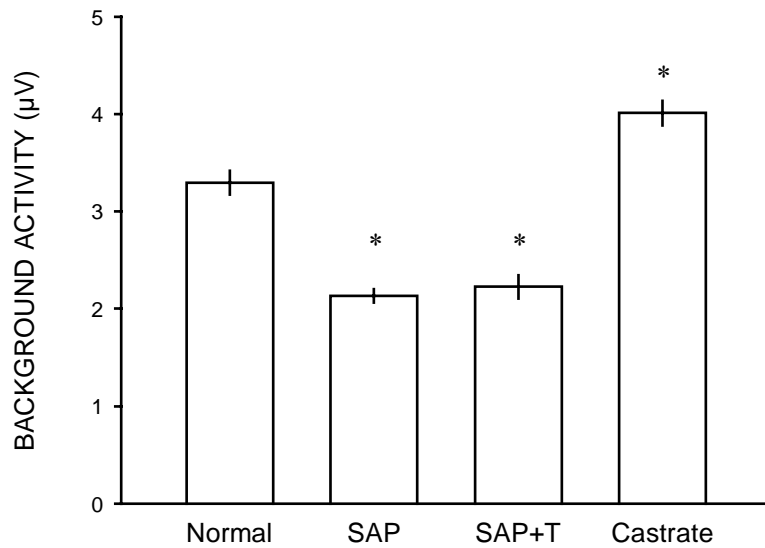


FIGURE 4.5

Average background activity for normal controls, untreated saporin-injected animals, saporin-injected animals treated with testosterone, and castrated controls, with the outlier removed from the untreated saporin-injected group. Saporin injection decreased average background activity, and treatment with exogenous testosterone did not prevent this decrease. Castration alone increased average background activity. \* indicates significantly different from normal controls,  $p < .001$ . Bar heights represent means  $\pm$  *SEM*.

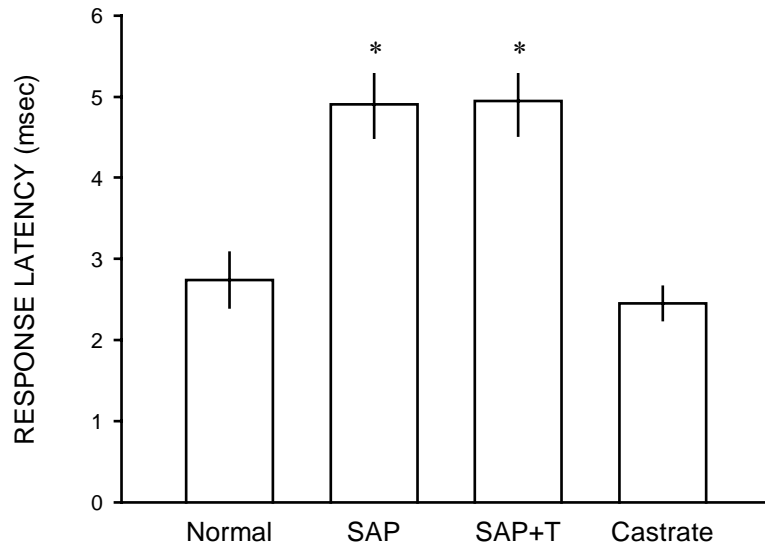


FIGURE 4.6

Response latencies for normal controls, untreated saporin-injected animals, saporin-injected animals treated with testosterone, and castrated controls. Saporin injection increased response latency, and treatment with exogenous testosterone did not prevent this. Castration alone had no effect on response latency. \* indicates significantly different from normal controls,  $p < .001$ . Bar heights represent means  $\pm$  SEM.

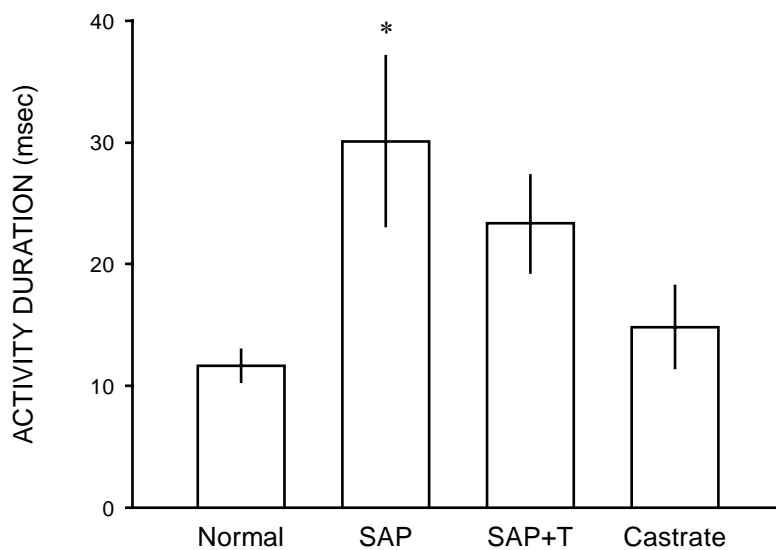


FIGURE 4.7

Activity durations for normal controls, untreated saporin-injected animals, saporin-injected animals treated with testosterone, and castrated controls. Saporin injection markedly increased activity duration. While the activity duration of testosterone-treated saporin-injected animals was not significantly different from that of normal controls, it was clearly closer to that of untreated saporin-injected animals. Castration alone had no effect on activity duration. \* indicates significantly different from normal controls,  $p < .01$ . Bar heights represent means  $\pm$  SEM.

saporin-injected animal had an activity duration of less than 17 msec. In saporin-injected animals treated with testosterone, activity duration was intermediate ( $23.32 \pm 3.99$  msec): while it was not significantly different from normal controls (LSD,  $p = .08$ ), it clearly had more overlap with untreated saporin-injected animals. Activity duration in castrated controls ( $14.82 \pm 3.36$  msec) did not differ from normal controls (LSD, *ns*). Activity duration is defined as the amount of time between the first and last spikes, yet while there were significant group differences in activity duration, there weren't any group differences in the number of spikes produced. High intensity stimulation resulted in an average of  $12.66 \pm 1.21$  spikes, and this did not differ between groups [ $F(3, 16) = 1.21$ , *ns*].

Maximum peak-to-peak response amplitude averaged  $2.66 \pm 0.49$  mV, and this did not differ between groups [ $F(3, 17) = 0.40$ , *ns*]. Additionally, maximum peak-to-peak response amplitude was examined across stimulus intensity. As is expected for a recruitment curve, peak-to-peak response amplitude increased significantly with stimulus intensity [ $F(3, 36) = 16.15$ ,  $p < .001$ ]. However, there was no effect of group [ $F(2, 12) = 0.33$ , *ns*] and no interaction between group and stimulus intensity [ $F(6, 36) = 0.70$ , *ns*].

The maximum response as measured by area under the curve averaged  $5.56 \pm 1.12$  mV/msec, and this did not differ between groups [ $F(3, 17) = 1.13$ , *ns*]. Response size measured as area under the curve was also examined across stimulus intensities. As is expected for a recruitment curve, area under the curve increased significantly with stimulus intensity [ $F(3, 36) = 28.23$ ,  $p < .001$ ]. While the statistical analysis did not indicate a significant effect of group [ $F(2, 12) = 0.79$ , *ns*] or an interaction between group and stimulus intensity [ $F(6, 36) = 0.40$ , *ns*], examination of the graphical representation

of the recruitment curve suggests an effect of group (see Figure 4.8). It appears that saporin injection depressed SNB motoneuron activation. Moreover treatment with testosterone appears to have attenuated this depression, specifically at levels of stimulus intensity above 61% of the maximally effective stimulus.

## DISCUSSION

### Muscle Weights

Saporin injection decreased BC/LA muscle weight on the injected side of the muscle complex (Figure 4.2). This is consistent with the results of my previous studies (Fargo and Sengelaub, 2004a, 2004b) and the experiments reported in Chapters 3 and 6 of this thesis. Testosterone treatment resulted in higher BC/LA weight on both the non-injected and saporin-injected sides of the muscle. This serves as an important manipulation check in this experiment, given that testosterone treatment failed to prevent several changes in motor activation caused by saporin injection.

This protective effect of testosterone treatment on muscle weight is not always seen following saporin injection. In one previous experiment (Fargo and Sengelaub, 2004a) testosterone treatment prevented atrophy of the saporin-injected muscles, while in another (Fargo and Sengelaub, 2004b) it did not. Given that the testosterone treatment was delayed in the former experiment, but immediate in the latter experiment, a reasonable question was whether the ability of testosterone to protect muscle weight following saporin injection was somehow tied to a delay between saporin injection and the onset of testosterone treatment. However, in the present experiment, testosterone treatment was immediate and still had a protective effect on muscle weight following saporin injection—this refutes the hypothesis that a delay between saporin injection and

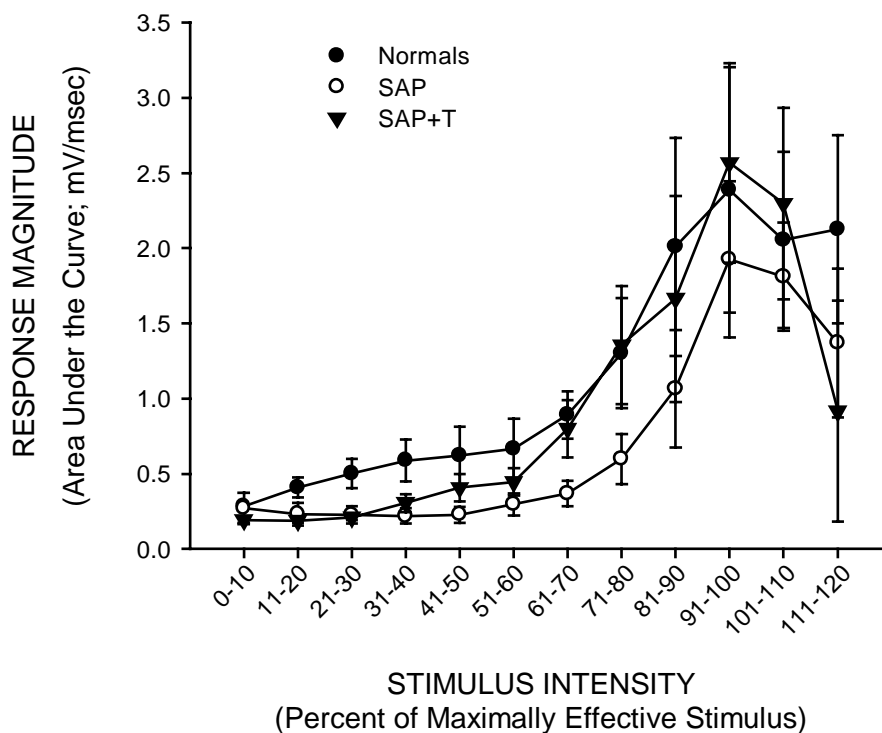


FIGURE 4.8

Recruitment curve illustrating the functional relationship between stimulus intensity and response magnitude. Data are shown for normal controls (filled circles), untreated saporin-injected animals (open circles), and saporin-injected animals treated with testosterone (triangles). Saporin injection attenuated SNB recruitment across the entire range of stimulus intensities. At high stimulus intensities, treatment with exogenous testosterone prevented this. Symbol heights represent means  $\pm$  SEM.

testosterone treatment is necessary in order for testosterone to be effective in protecting muscle weight.

### Background Activity

Average background activity was recorded for approximately 13 msec before each stimulus pulse was delivered. Background activity was significantly reduced in saporin-injected animals, and treatment with testosterone did not prevent this (Figure 4.5). In contrast, castration alone increased background activity significantly. This is the first ever report of experimental manipulation causing changes in SNB background activity.

Importantly, these data do not map neatly onto what is known about the effects of saporin injection and hormone treatments on SNB motoneuron morphology, in which castration and saporin injection produce similar regressive changes, and testosterone treatment prevents or reverses these changes. Several important conclusions can be drawn from this observation. First, although saporin injection and castration both lead to regressive morphological changes, each also results in its own unique changes to the SNB/BC system. This is not surprising in light of the fact that saporin injection kills a large portion of SNB motoneurons, whereas castration does not result in any cell death. Second, while this experiment grew out of the speculation that the regressive morphological consequences of saporin injection were likely to produce changes in SNB/BC electrophysiology, it is clear that saporin-induced changes in this system's electrophysiology cannot be explained exclusively by morphological changes. Otherwise, saporin injection and castration could not have similar effects on morphology but opposite effects on background activity. Finally, while testosterone treatment has



powerful protective effects against saporin-induced dendritic regression, at least some of the electrophysiological consequences of saporin injection are not amenable to testosterone treatment.

### Evoked Activity

In intact animals, the first activity spike began approximately 2.7 msec after the stimulus pulse was delivered. Saporin injection increased response latency to nearly 5 msec, and testosterone treatment did not prevent this increase in response latency (Figure 4.6). Castration alone had no effect on response latency. These data suggest that central response latency is not controlled by testosterone. This finding is in agreement with a previous report that castration and testosterone replacement have no effect on SNB nerve conduction velocities or central delay (Tanaka and Arnold, 1993). This stands in stark contrast to the effect of testosterone on response latency in the *peripheral* motor components of the SNB system. When SNB motor axons are stimulated in the periphery and EMG is recorded at the BC muscle, response latency is increased by castration, and treatment with gonadal steroids prevents this increase (Fargo et al., 2003; Foster and Sengelaub, 2004b). The response latency data presented here provide another example of saporin causing changes to the electrophysiological properties of the SNB/BC system that are independent of its effects on morphology and not amenable to testosterone treatment.

The duration of activity was defined as the amount of time between the beginning of the first activity spike and the end of the last activity spike. Saporin injection increased activity duration, and testosterone did not effectively prevent this (Figure 4.7). Castration alone had no effect on activity duration. In contrast to activity duration, the actual number of spikes produced in response to stimulation was not different between groups,

suggesting that the activity was more spread out in the saporin-injected groups. Only two other reports of SNB activity duration or spike counts have been made. In the first, McKenna and Nadelhaft (1989) reported that non-spinalized animals exhibited what they called “later components” of the reflex, which appear to correspond to the spikes occurring late in the activity duration of animals in my saporin-injected groups. They reported, however, that these were invariably abolished by spinal transection. Given that all of my animals were spinally transected, it is difficult to judge exactly how the “later components” of McKenna and Nadelhaft relate to the increase in activity duration seen in my saporin-injected animals. In the second report (Fargo et al., 2003), the number of spikes in response to stimulation was decreased by castration, but maintained by concurrent treatment with estradiol. However, the experimental preparation in that study utilized a modified H-reflex setup, and spikes were counted for high-intensity stimulations. In the H-reflex setup, which involves both orthodromic and antidromic stimulation of motor axons, high-intensity stimulations tend to suppress reflex activity via afterhyperpolarization of the motoneurons. It is therefore possible that the reduced spike count associated with steroid withdrawal in that experiment was due to hormonal modulation of the *inhibition* of activity, rather than modulation of the underlying excitability of the system at rest. In light of the fact that castration had no effect on either activity duration or spike count in the current experiment, this seems like the most likely explanation.

Maximal responses, as measured by either peak-to-peak amplitude or area under the curve, did not differ between groups. However, the recruitment curve plotting response magnitude (as measured by area under the curve) against stimulus intensity

reveals that SNB recruitment is depressed by saporin injection, and that testosterone treatment prevents this depression at higher levels of stimulus intensity (Figure 4.8). Taken together, these data suggest that untreated saporin-injected animals maintain their ability to produce maximal responses, but the likelihood that they will produce a high magnitude response to any given stimulus is markedly decreased. Testosterone treatment, while not increasing the maximum response possible, increases the likelihood that the saporin-injected animal will produce a high magnitude response to a given stimulus.<sup>1</sup>

#### Functional correlate of testosterone's protective effect on anatomy?

A clear pattern emerged in the electrophysiological data from this experiment: Saporin injection led to changes in several measures of central SNB electrophysiology; however, these changes were not prevented by hormone treatment. Saporin depressed average background activity, and increased both response latency and activity duration, and testosterone treatment had no effect on these measures. In fact, most of these measures seemed to be completely impervious to testosterone manipulation, as castration alone also had no effect on them (the lone exception is background activity, which is slightly increased by castration). This pattern is completely different from that seen in the morphology of SNB motoneurons: saporin injection and castration alone both lead to somal and dendritic atrophy, and testosterone treatment prevents these regressive changes. Therefore, there is no simple relationship between these measures of SNB motor function and testosterone's protective effect on SNB morphology.

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<sup>1</sup> It is noteworthy that testosterone appears to be most effective in protecting SNB recruitment at high stimulus intensities and correspondingly high response magnitudes. Penile reflexes during copulatory behavior are mediated by a series of very strong contractions of the BC/LA muscle complex (Hart and Melese-D'Hospital, 1983). Therefore, protection of high response magnitudes would be expected to be more functionally relevant than protection of lower response magnitudes.

Of course, it is highly unlikely that shrunken contralateral somata and dendritic arbors are the only neuroanatomical differences between normal controls and saporin injected animals. Saporin injection results in the death of ipsilateral motoneurons, and may also kill ipsilateral primary afferents. In addition, these losses are likely to result in the reorganization of other inputs into the surviving SNB motoneurons, such as those coming from interneurons. This hypothetical reorganization of the spinal reflex circuitry would be expected to give rise to changes to the electrophysiological response properties of the reflex, such as the changes in background activity, response latency, and activity duration reported here. Some of these hypothetical changes in neuroanatomy may not respond to testosterone treatment, and the electrophysiological changes arising from them would therefore also be expected to be unresponsive to testosterone treatment.

At least one measure of SNB electrophysiology, however, did appear to correlate with SNB somal and dendritic morphology following saporin injection. Saporin injection decreased SNB recruitment, and testosterone treatment prevented this decrease. This pattern of results exactly mirrors the protective effect of testosterone on the morphology of SNB motoneuron somata and dendrites following saporin injection. This suggests the possibility that recruitment is mediated at least in part by motoneuron morphology. One can easily imagine the increased surface area of larger somata and dendritic arbors providing sites for additional afferent input. However, based solely on the present data, one cannot conclude that SNB morphology directly impacts recruitment—only that the two change in concert following saporin injection and testosterone treatment.

## Conclusions

The purpose of this experiment was to determine whether any electrophysiological changes accompany SNB motoneuron atrophy induced by contralateral saporin injection, and moreover, to determine whether treatment with exogenous testosterone would prevent these changes as it prevents motoneuron atrophy. Several measures of SNB electrophysiology were affected by saporin injection: average background activity, response latency, activity duration, and recruitment. Testosterone treatment restored recruitment to normal levels. These data indicate that the atrophy of SNB motoneurons caused by saporin injection is indeed accompanied by changes in their functional electrophysiology. More importantly, they demonstrate that testosterone treatment, in addition to restoring normal morphology, restores the ability of the system to produce normal responses at functionally relevant stimulation levels.

## Chapter 5.

Does Testosterone Protect against Regressive Changes in Motoneurons following Partial Motoneuron Depletion Induced by Ventral Root Avulsion?

In previous experiments (Fargo and Sengelaub, 2004a, 2004b) I depleted SNB motoneurons by injection of a retrogradely transported toxin into their target musculature. This method has several advantages in terms of experimental control. First, it is neurotoxic regardless of hormone status (Fargo and Sengelaub, 2004a, 2004b). This is an important consideration because testosterone has been shown to protect neurons from cell death in several experimental paradigms. For example, testosterone prevents the death of cultured neurons following serum deprivation (Hammond et al., 2001), oxidative stress (Ahlbom et al., 2001), or exposure to  $\beta$ -amyloid (Pike, 2001); and *in vivo*, testosterone prevents neuron death in kainite-induced lesions (Ramsden et al., 2003). Because I am interested in testosterone's protective effects on surviving motoneurons after depletion, it is important that my method of motoneuron depletion be effective even when the animal is treated with exogenous testosterone. Second, saporin injection is laterally specific, killing motoneurons only on the side of the spinal cord ipsilateral to the injected musculature (Fargo and Sengelaub, 2004a, 2004b). Saporin injection results in ipsilateral motoneuron depletion within the first week post-injection, and the number of surviving motoneurons does not change over the course of the next three weeks (Fargo and Sengelaub, 2004a), thus making it unlikely that contralateral SNB motoneurons are in the process of dying. That means that they can be used to determine what happens to the morphology of motoneurons whose counterparts have been depleted, but who are not themselves in the process of dying.

However, motoneuron depletion by saporin injection also has a potential drawback in that I am introducing a neurotoxin into the spinal cord. This raises the possibility that the morphological changes seen contralateral to the depletion are due to a

direct effect of saporin on those contralateral cells (or some other action of saporin in the spinal cord), instead of being due to the depletion of the ipsilateral cells. While the fact that motoneuron number stays constant contralaterally suggests that saporin is not *killing* contralateral motoneurons, it does not rule out the possibility that saporin is affecting them in some other way. Consequently, the protective effect of testosterone might be specific to situations in which a toxin is introduced into the spinal cord.

In order to test the generalizability of testosterone's protective effect, it would be desirable to deplete motoneurons without the use of an exogenous neurotoxin<sup>1</sup> and then treat with testosterone. In adult animals it is possible to kill substantial number of motoneurons by ventral root avulsion (VRA; Hoffmann and Thomeer, 1992; Koliatsos et al., 1994). In VRA, the ventral roots are physically separated from the spinal cord by pulling them away. This ruptures axons near or inside the spinal cord, puts the somata under physical strain, and results in the death of the majority of motoneurons whose axons occupy the avulsed roots. Therefore, VRA offers a laterally specific method to kill motoneurons without introducing an exogenous neurotoxin. Using VRA, then, can eliminate the possibility that the morphological changes seen in surviving motoneurons, and therefore the protective effect of testosterone, are related to the presence of an exogenous toxin.

## METHODS AND DESIGN

Adult male Sprague Dawley rats were used. Animals in the VRA groups were placed under general anesthesia with an intraperitoneal injection of Chlorapent (0.3 ml/100 g body weight). Once animals no longer responded to pain, they were placed in

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<sup>1</sup> In developing animals this can be accomplished by muscle extirpation (Goldstein et al., 1993), but this method does not work in adults (e.g., Wu et al., 2003).



the prostrate position, and an incision of approximately 10 cm was made through the skin directly over, and parallel to, the spine. The skin was retracted with ophthalmic spreaders, and a scalpel was used to cut through the paraspinous muscles and connective tissue on the right side until the lateral aspect of the 2<sup>nd</sup> lumbar vertebra was completely exposed. The muscles and connective tissue were retracted with ophthalmic spreaders, and a few minutes were allowed to pass so that bleeding would subside before exposing the central nervous tissue. I then used rongeurs to create a small window approximately 4 to 5 mm long and 2 to 3 mm high in the right side of the 2<sup>nd</sup> lumbar vertebra. When this caused significant bleeding, the procedure was paused long enough for the bleeding to subside before continuing. The dural sheath was then cut along the length of the window, exposing the right side of the spinal cord and the right dorsal and ventral roots. The right ventral roots L5-S1 (which carry the SNB motoneurons' axons) were located, and each was avulsed by the application of gentle traction caudally in a direction roughly parallel to their natural course. Once the ventral roots were separated from the spinal cord, a length of at least 1 cm of the distal stump was removed from each in order to prevent reinnervation. The window was then covered with a small piece of gelfoam to prevent the entry of blood into the cerebrospinal fluid. The muscles and connective tissue were then closed with silk sutures, and the skin closed with 9-mm AutoClips brand stainless steel wound clips. The animal was then allowed to recover from anesthesia. My technique is relatively unobtrusive compared to other root avulsion procedures, which are much more extensive yet have still been used without problem for survival times ranging up to several months (Blits et al., 2004; Gu et al., 2004; Gu et al., 2005; Hoang et al., 2003; Kishino et al., 1997).

Coincident with ventral root avulsion, some animals were also simultaneously castrated and given replacement testosterone according to the methods described in Chapter 2 (VRA only  $n = 6$ , VRA and testosterone treatment  $n = 5$ ). For the testosterone-treated animals, a testosterone implant was placed under the skin after closing the muscles and connective tissue but before closing the skin. A group of untreated intact normal males ( $n = 11$ ) and a group of untreated castrates ( $n = 8$ ) served as control groups. Four weeks after VRA and the onset of testosterone treatment, I injected HRP into the BC muscle on the left side (contralateral to the motoneuron depletion). The HRP injection was carried out according to the method described in Chapter 2. After two additional days to allow for optimal HRP transport, animals were killed and the perineal musculature removed and weighed according to the methods described in Chapter 2. Lumbosacral spinal cords were removed by laminectomy rather than the method described in Chapter 2, because laminectomy is less likely to damage the spinal roots. This allowed me to inspect each spinal cord to ensure the complete avulsion of ventral roots L5-S1. Spinal cords were then trimmed and processed for motoneuron counts and morphometry according to the methods described in Chapter 2.

Rostral dendritic extent was assessed by counting the number of sections between the most rostral HRP-labeled process and the most rostral HRP-labeled cell body. As the centers of these sections are  $40\ \mu\text{m}$  apart, the number of sections was multiplied by  $40\ \mu\text{m}$  to arrive at an estimate of the actual linear distance spanned. Caudal dendritic extent was assessed in a similar fashion at the caudal end of the nucleus.

## RESULTS

### Muscle Weights

The weight of the BC/LA muscle complex differed between groups on both the left (HRP-injected) and right (VRA) sides [left side  $F(3, 15) = 57.59, p < .001$ ; right side  $F(3, 15) = 97.45, p < .001$ ; see Figure 5.1]. Ventral root avulsion resulted in a drastic reduction in ipsilateral BC/LA weight ( $0.207 \pm 0.016$  g for VRA animals compared to  $0.606 \pm 0.0301$  g for normal controls; LSD,  $p < .001$ ), and this was not prevented by testosterone treatment ( $0.300 \pm 0.016$  g for VRA animals treated with testosterone; compared to normal controls, LSD,  $p < .001$ ). Muscle atrophy induced by ventral root avulsion was specific to the avulsed side (left side BC/LA muscle weights:  $0.540 \pm 0.022$  g for VRA animals, and  $0.656 \pm 0.034$  g for VRA animals treated with testosterone, compared to  $0.612 \pm 0.030$  g for normal controls; LSDs, *ns*). Notably, treatment of VRA animals with testosterone increased BC/LA muscle weight on both sides of the animal (compared to VRA animals, LSD,  $ps < .01$ ). Consistent with the previous literature (Eisenberg et al., 1949; Fargo and Sengelau, 2004a; Wainman and Shipounoff, 1941), castration drastically reduced BC/LA weight ( $0.194 \pm 0.007$  g; compared to normal controls, LSD,  $p < .001$ ).

### Cell Counts

Ventral root avulsion resulted in the death of over 65% of ipsilateral SNB motoneurons [ $38.00 \pm 6.26$  remaining motoneurons for VRA animals compared to  $108.80 \pm 11.34$  for normal controls, LSD,  $p < .001$ ; overall test for the effect of group on motoneuron number  $F(3, 15) = 32.90, p < .001$ ; see Figure 5.2], and treatment with testosterone did not prevent this ( $20.80 \pm 3.67$  remaining motoneurons for VRA animals

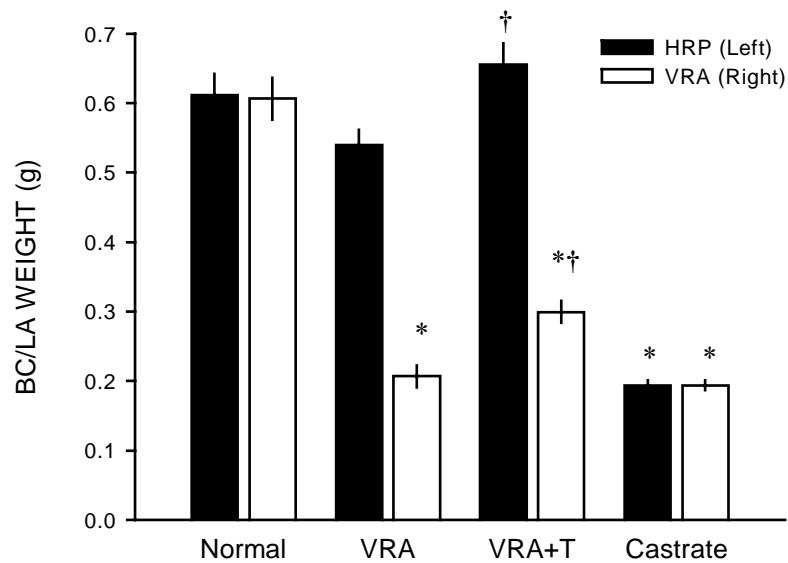


FIGURE 5.1

BC/LA muscle weights for normal controls, VRA animals, testosterone-treated VRA animals, and castrated controls, for both the root-avulsed side (open bars) and the non-avulsed side (filled bars). Ventral root avulsion caused marked muscle atrophy, which was specific to the avulsed side and was not prevented by testosterone treatment. \* indicates significantly different from normal controls,  $p < .001$ . † indicates significantly different from VRA animals,  $p < .01$ . Bar heights represent means  $\pm$  SEM.

Note: For normal controls and castrated controls, the open bars represent the right side and the filled bars represent the left side of the BC/LA muscle complex.

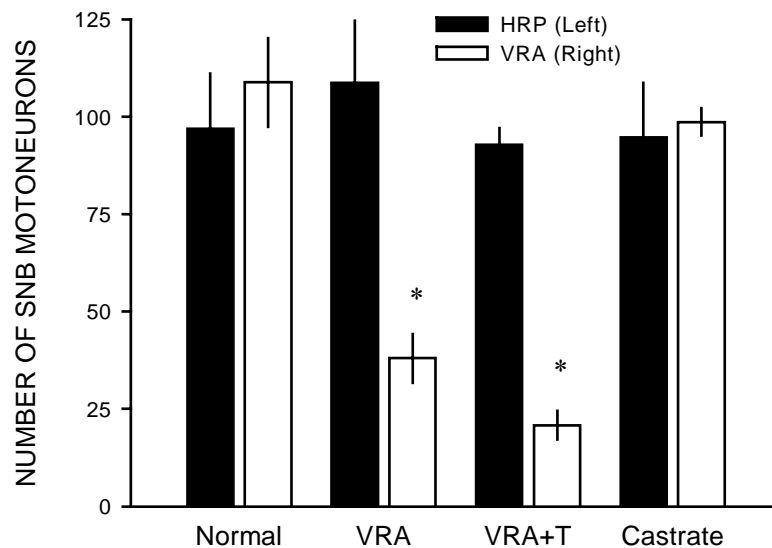
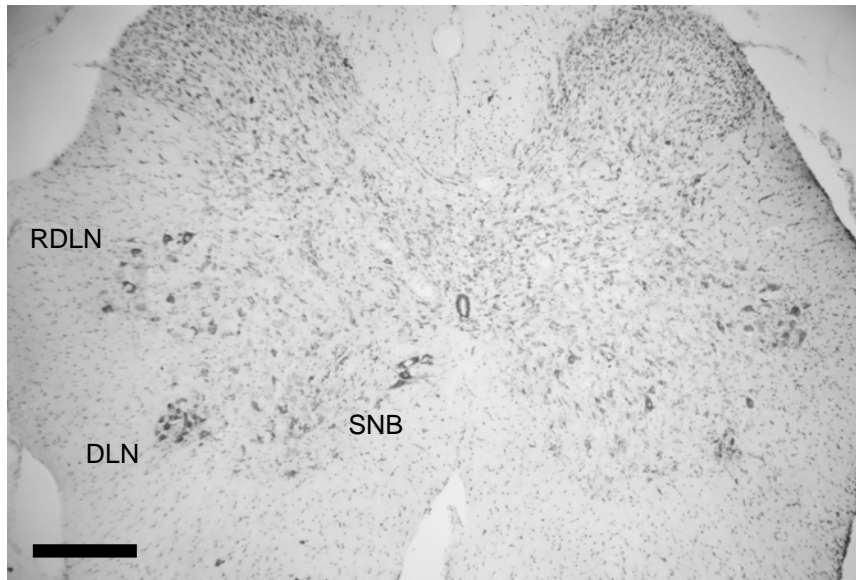


FIGURE 5.2

**Top:** Brightfield photomicrograph of a transverse section through the lumbar spinal cord of a testosterone-treated VRA animal four weeks after unilateral avulsion of ventral roots L5-S1, showing three bilateral lumbosacral spinal nuclei: the retrodorsolateral nucleus (RDLN), the dorsolateral nucleus (DLN), and the SNB. Note the dearth of motoneurons on the avulsed (right) side of the spinal cord. Scale bar = 250  $\mu$ m. **Bottom:** Numbers of thionin-stained SNB motoneurons in normal controls, VRA animals, testosterone-treated VRA animals, and castrated controls, for both the root-avulsed side (open bars) and the non-avulsed side (filled bars). Ventral root avulsion killed more than 65% of the ipsilateral SNB motoneurons, regardless of hormone status, and did not kill contralateral motoneurons. \* indicates significantly different from normal controls,  $p < .001$ . Bar heights represent means  $\pm$  SEM.

Note: For normal controls and castrated controls, the open bars represent the right half and the filled bars represent the left half of the SNB.

treated with testosterone; compared to normal controls, LSD,  $p < .001$ ). Consistent with the previous literature (Breedlove and Arnold, 1981; Fargo and Sengelaub, 2004a, 2004b), castration did not result in the death of SNB motoneurons ( $98.67 \pm 3.53$  remaining motoneurons for castrated controls; compared to normal controls, LSD, *ns*); the numbers of SNB motoneurons in both castrated and normal controls were well within the normal range. Importantly, SNB motoneuron death induced by ventral root avulsion was specific to the ipsilateral side: on the contralateral side, the average number of remaining motoneurons was  $99.16 \pm 6.41$ , and this did not differ between groups [ $F(3, 15) = 0.32$ , *ns*]

### Morphometry

#### Somata

Soma areas for normal controls were  $1068.71 \pm 57.88 \mu\text{m}^2$ . Castrated controls had somata of significantly smaller area, demonstrating the sensitivity of the measure ( $819.85 \pm 55.29 \mu\text{m}^2$  for castrated controls; compared to normal controls, LSD,  $p < .01$ ; overall test for the effect of group on soma area  $F(3, 20) = 5.02$ ,  $p < .01$ ; see Figure 5.3).

However, neither VRA animals nor VRA animals treated with testosterone differed from normal controls in their soma areas ( $1030.66 \pm 54.33 \mu\text{m}^2$  for VRA animals and  $1033.88 \pm 41.31 \mu\text{m}^2$  for VRA animals treated with testosterone; LSDs, *ns*). Somata were traced for an average of  $21.14 \pm 0.61$  HRP-labeled motoneurons per animal, and this did not differ between groups [ $F(3, 17) = 1.60$ , *ns*].

$\chi^2$  analysis of individual soma areas yielded similar results (see Figure 5.4).

Castration shifted the distribution of individual soma areas toward the smaller end of the continuum [compared to normal controls,  $\chi^2(11) = 45.99$ ,  $p < .001$ ; overall test for the

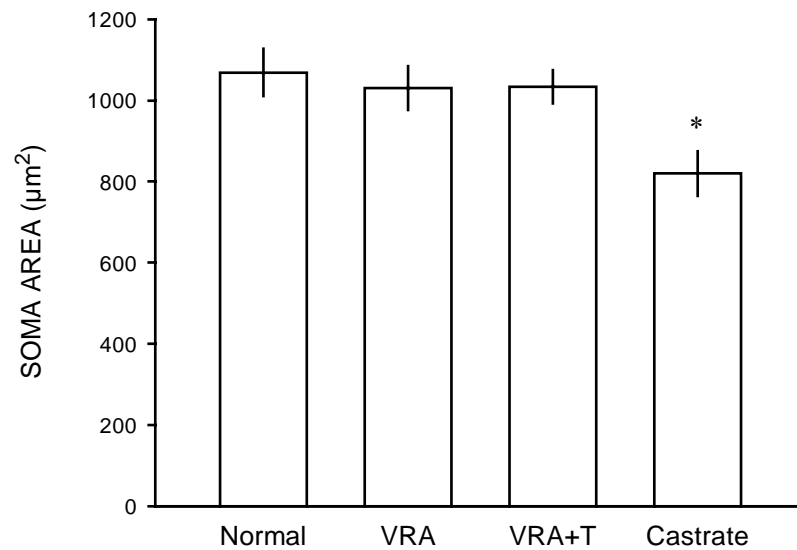


FIGURE 5.3

Soma areas of SNB motoneurons of normal controls, VRA animals, testosterone-treated VRA animals, and castrated controls. Castrated controls had smaller somata than the other groups, but neither VRA animals nor VRA animals treated with testosterone differed from normal controls. \* indicates significantly different from normal controls,  $p < .001$ . Bar heights represent means  $\pm$  SEM.

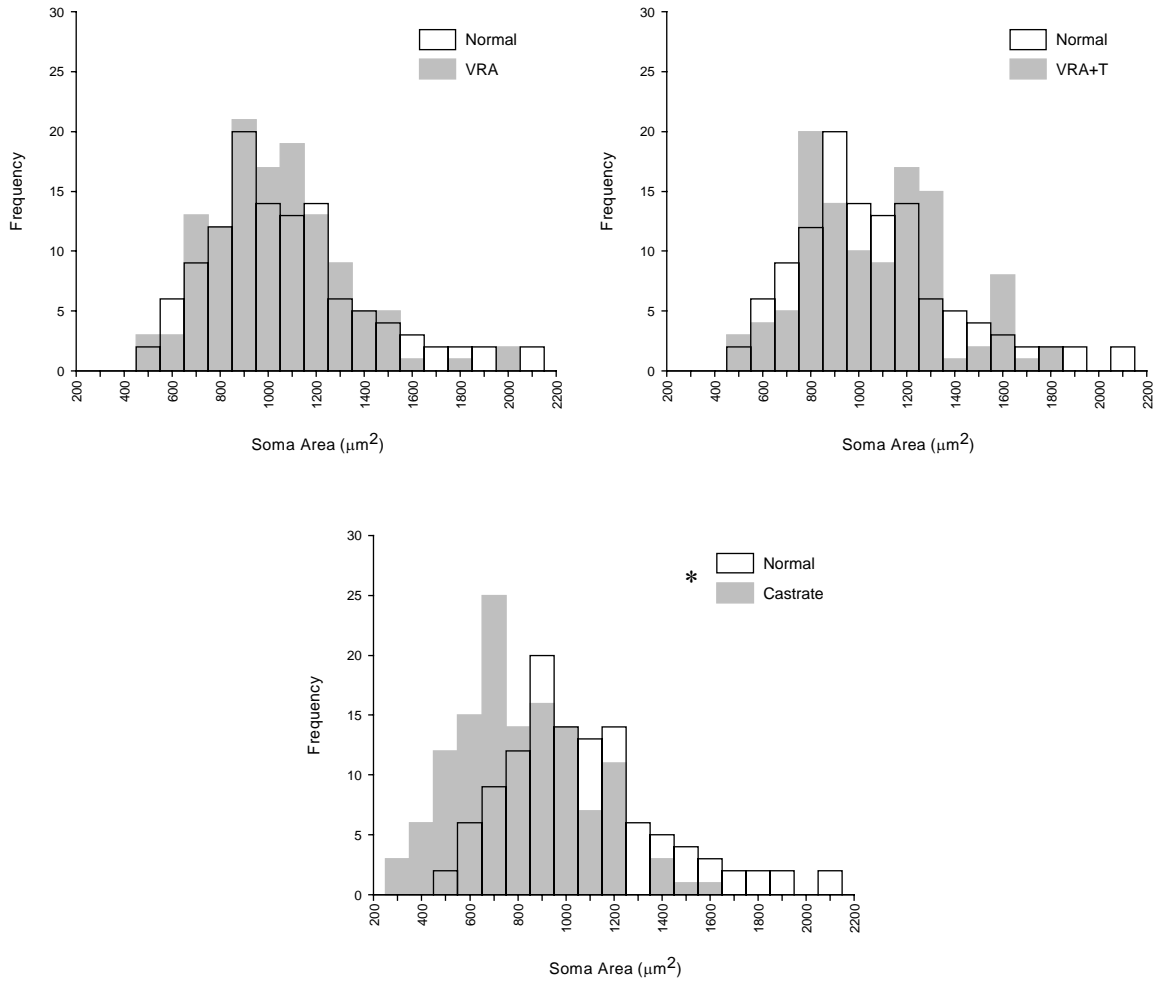


FIGURE 5.4

Frequency distributions of individual soma areas of BC-projecting SNB motoneurons for normal controls, VRA animals, testosterone-treated VRA animals, and castrated controls. Only castrated controls had a frequency distribution different from that of normal controls, with castration shifting the distribution toward smaller somata. \* indicates significantly different from normal controls,  $p < .001$ .



effect of group on the distribution of individual soma areas  $\chi^2(33) = 109.75, p < .001$ ].

However, the frequency distributions of soma areas for VRA animals and VRA animals treated with testosterone did not differ from that of normal controls [ $\chi^2(11) \leq 13.57, ns$ ].

### Dendrites

Injection of HRP into the left BC successfully labeled ipsilateral SNB motoneurons in a manner consistent with previous studies (Fargo and Sengelaub, 2004a, 2004b; Kurz et al., 1991; Kurz et al., 1986). SNB motoneurons displayed their characteristic multipolar morphologies (Kurz et al., 1986; Sasaki and Arnold, 1991), with dendritic arbors projecting ventrolaterally, dorsomedially, and across the midline into the area of the contralateral SNB (Figure 5.5). An average of  $78.10 \pm 4.56$  motoneurons per animal were labeled with HRP.

Dendritic length was an average of  $5954.43 \pm 640.11 \mu\text{m}$ , and group differences did not reach significance [ $F(3, 17) = 2.83, ns$ ]. However, dendritic lengths for castrated controls ( $3145.60 \pm 246.27 \mu\text{m}$ ) were much smaller than for any other group<sup>2</sup>, and the dendritic lengths for the other three groups were very similar ( $7694.93 \pm 1728.55 \mu\text{m}$  for normal controls,  $6321.77 \pm 1018.61 \mu\text{m}$  for VRA animals, and  $6581.96 \pm 1063.48 \mu\text{m}$  for VRA animals treated with testosterone; see Figure 5.6). In any case, the important result here is that the testosterone-treated animals are certainly no better off than the untreated VRA animals.

Dendritic length was nonuniform across radial bins, and repeated-measures ANOVA revealed a significant effect of radial location [ $F(11, 187) = 108.18, p < .001$ ]. Consistent with the results of the arbor per cell analysis, there was no significant effect of

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<sup>2</sup> In fact, comparing only the normal controls and castrated controls suggests that castration did indeed cause a reduction in dendritic length [ $t(8) = 2.61, p < .05$ ].

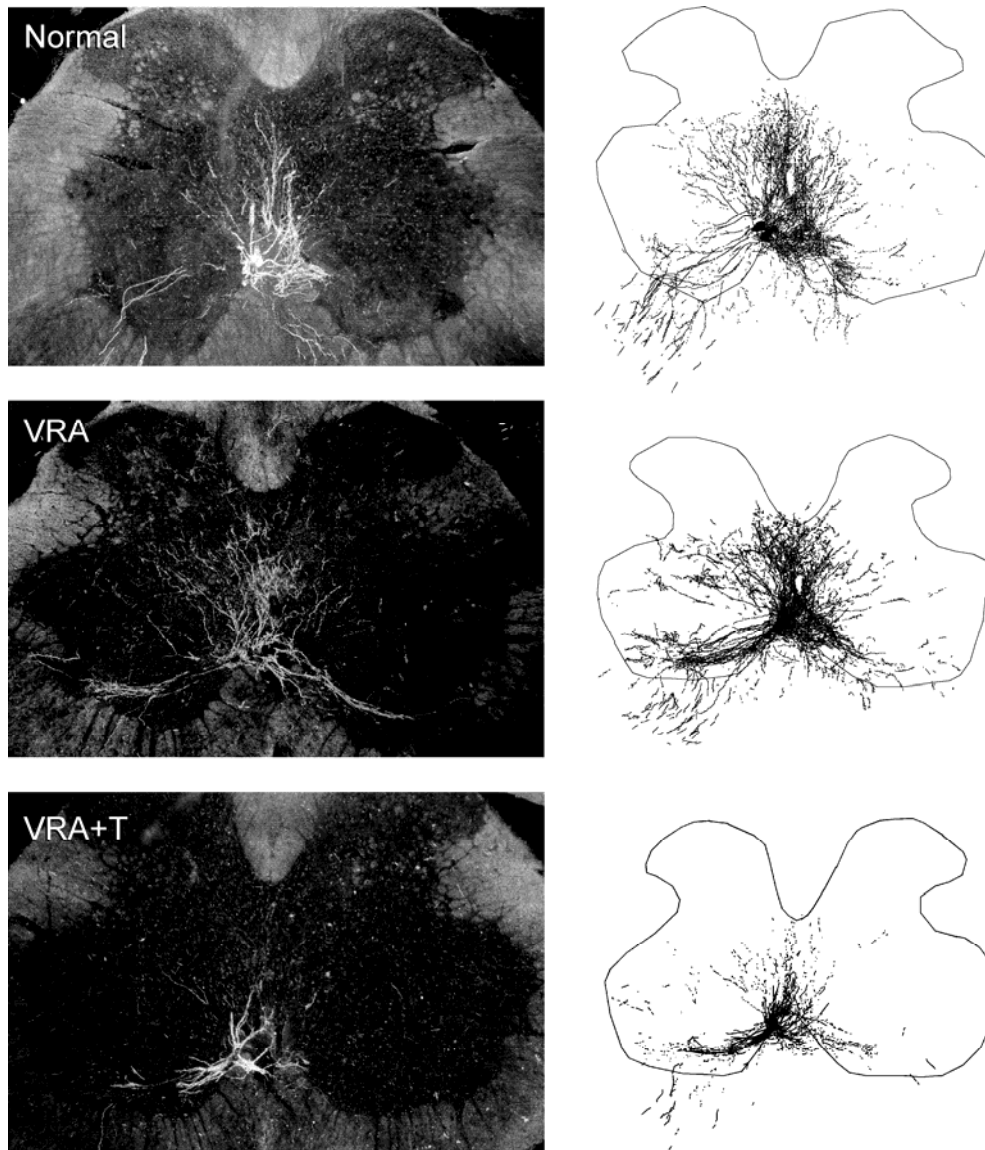


FIGURE 5.5

Left: Darkfield photomicrographs of transverse sections through the lumbar spinal cords of a normal control, a VRA animal, and a castrated control, after HRP injection into the left BC muscle. Right: Computer-generated composites of HRP-labeled somata and processes drawn at 320  $\mu\text{m}$  intervals through the entire rostrocaudal extent of the SNB; these composites were selected because they are representative of their respective group average dendritic lengths.

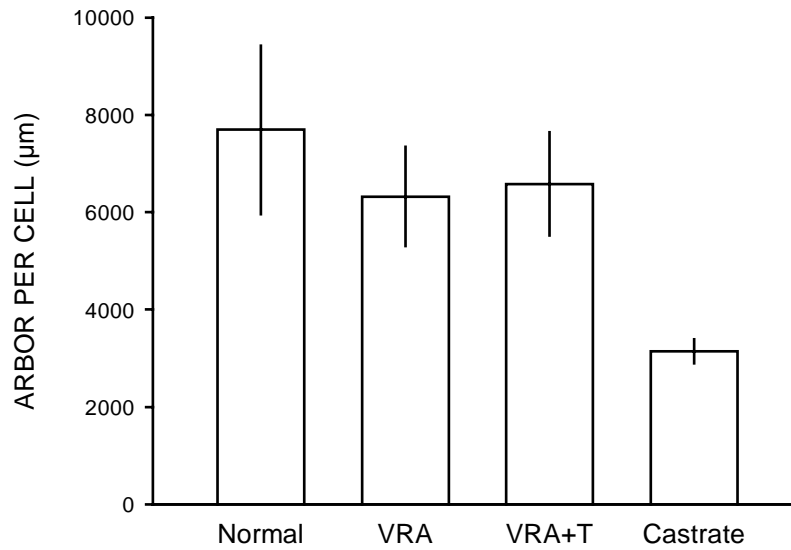


FIGURE 5.6

Dendritic lengths of SNB motoneurons of normal controls, VRA animals, testosterone-treated VRA animals, and castrated controls. Although group differences did not reach statistical significance, a visual examination of the data indicates that castrated controls had shorter dendritic arbors than any other group, and that neither ventral root avulsion nor testosterone treatment had any effect on dendritic length. Bar heights represent means  $\pm$  SEM.

group [ $F(3, 17) = 2.83, ns$ ], although castrated animals had smaller dendritic lengths than every other group in every radial bin and the other groups were all fairly similar. Specifically, castration reduced the length of the dendritic arbor by 65% from 0° to 30°, 73% from 30° to 60°, 86% from 60° to 90°, 82% from 90° to 120°, 72% from 120° to 150°, 56% from 150° to 180°, 46% from 180° to 210°, 43% from 210° to 240°, 50% from 240° to 270°, 58% from 270° to 300°, 62% from 300° to 330°, and 75% from 330° to 360° (see Figure 5.7). There was also a significant interaction between radial location and group [ $F(33, 187) = 2.69, p < .001$ , see Figure 5.7]; although there was no main effect of group, the dendritic lengths of VRA animals were shorter than those of normal controls in the most ventromedial portion of the spinal cord (240° to 300°), and testosterone treatment did not prevent this change.

Radial dendritic extent was nonuniform across bins, and repeated-measures ANOVA revealed a significant effect of radial location [ $F(11, 187) = 96.42, p < .001$ ]. However, radial dendritic extent was not affected by VRA, testosterone treatment, or castration: it did not differ between groups [ $F(3, 17) = 1.60, ns$ ], and there was no interaction between radial location and group [ $F(33, 187) = 0.80, ns$ ; see Figure 5.8]. Similarly, both rostral and caudal dendritic extent were unaffected by VRA, testosterone treatment, or castration [rostral  $F(3, 17) = 0.65, ns$ ; caudal  $F(3, 17) = 0.62, ns$ ].

## DISCUSSION

### Muscle Weights

Ventral root avulsion resulted in severe atrophy of the BC/LA musculature (see Figure 5.1). Notably, this atrophy was seen only on the side of the animal that received the avulsion, indicating that this manipulation is laterally specific in the periphery. This is

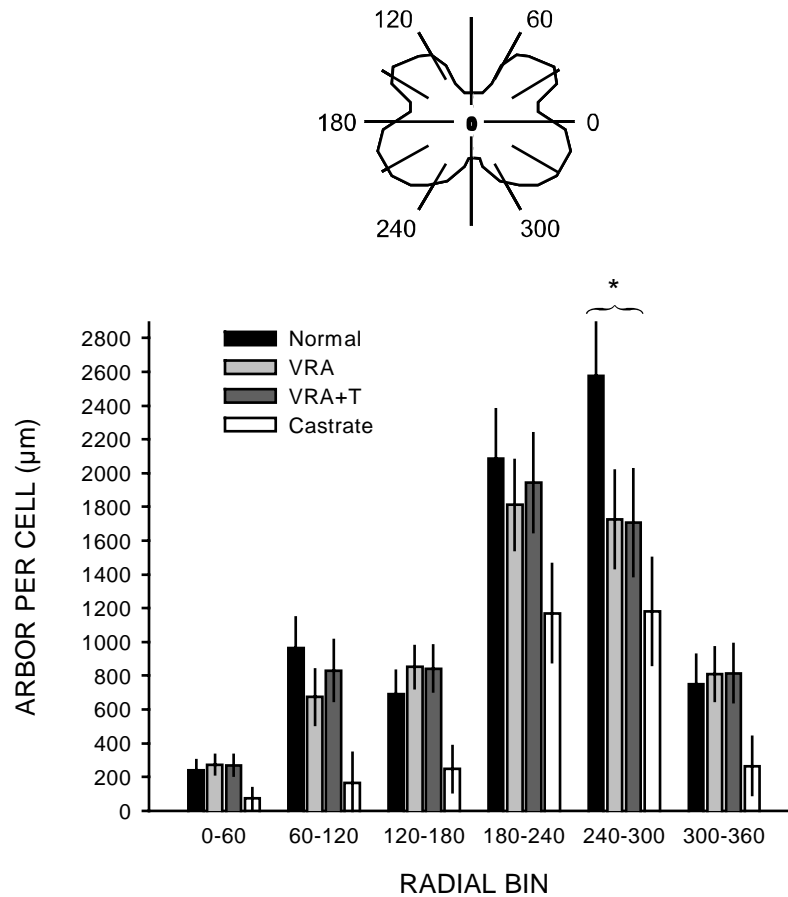


FIGURE 5.7

Top: Drawing of spinal gray matter divided into radial sectors for measure of SNB dendritic distribution. Bottom: Length per radial bin of SNB dendrites in normal controls, VRA animals, testosterone-treated VRA animals, and castrated controls. For graphic purposes, dendritic length measures have been collapsed into 6 bins of 60° each. SNB motoneuron dendritic arbors display a non-uniform distribution, with the majority of the arbor located between 180° and 300°. Although not reaching statistical significance, castration reduced dendritic length in every radial bin. In VRA animals, both untreated and testosterone-treated, dendritic length was reduced in the most ventromedial portion of the spinal cord (between 240° and 300°). \* indicates major source of significant interaction between group and radial bin,  $p < .001$ . Bar heights represent means  $\pm$  SEM.

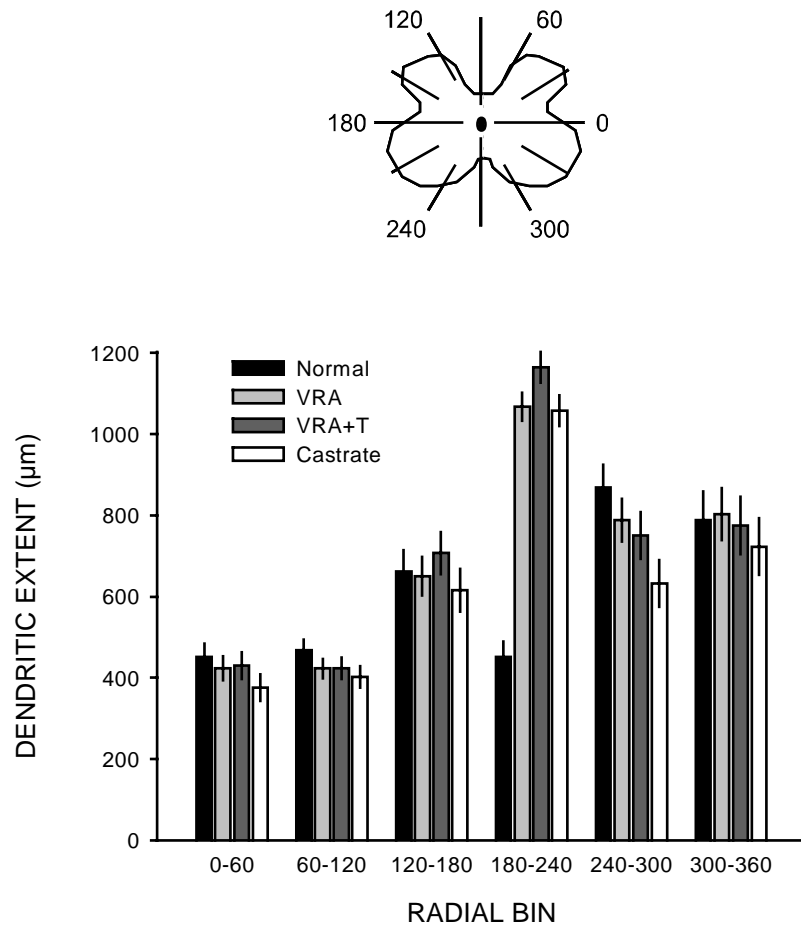


FIGURE 5.8

Top: Drawing of spinal gray matter divided into radial sectors for measure of SNB radial dendritic extent. Bottom: Radial extents of SNB dendrites in normal controls, VRA animals, testosterone-treated VRA animals, and castrated controls. For graphic purposes, dendritic extent measures have been collapsed into 6 bins of 60° each. SNB dendritic extent is non-uniform across radial bins, and this non-uniform distribution is apparent in all groups. Bar heights represent means  $\pm$  SEM.

important in establishing that any potential motoneuron atrophy on the intact side is not due simply to an effect of VRA on that side of the musculature. Castration also resulted in severe atrophy of the BC/LA muscles.<sup>3</sup>

While testosterone treatment was not capable of fully restoring the weight of the muscles following VRA, testosterone-treated VRA animals had heavier muscles (on both sides) than untreated VRA animals. Rand and Breedlove (1992) reported that exogenous testosterone was ineffective in maintaining the weight of the denervated BC and LA. However, the data I report here suggest that their conclusion was premature—testosterone can still affect the BC/LA musculature, even when they are disconnected from their innervating motoneurons. Moreover, testosterone also led to BC/LA hypertrophy on the intact side of the animal, again raising the possibility that testosterone treatment exerts its beneficial effects by acting through the musculature (but see Chapter 6).

### Cell Counts

The purpose of the present experiment was to unilaterally deplete SNB motoneurons using a method that does not introduce an exogenous neurotoxin into the spinal cord, in order to determine whether the beneficial effects of testosterone generalize to those situations; I chose to accomplish this end by avulsing the ventral roots L5-S1. Ventral root avulsion resulted in the death of over 65% of the motoneurons in the ipsilateral SNB (Figure 5.2). This amount of motoneuron death is on par with that seen after saporin injection into the BC and LA (Fargo and Sengelaub, 2004a, 2004b; Chapter 6), making VRA suitably lethal for the purposes of this experiment. Further, VRA-

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<sup>3</sup> It is a demonstration of just how dependent these muscles are on androgens that castration resulted in almost the same level of atrophy as did complete denervation by VRA.

induced motoneuron death was specific to the side of the animal that underwent the avulsion—no motoneurons contralateral to the avulsion died. This means that we can examine the morphology of motoneurons whose contralateral neighbors have been depleted, but who are not themselves in the process of dying.

In order to interpret the results in terms of a potential beneficial effect of testosterone on motoneuron morphology following partial motoneuron depletion, it was critical that VRA result in ipsilateral motoneuron death even in the presence of exogenous testosterone. In some injury paradigms, treatment with exogenous steroids prevents motoneuron death ( e.g., Ahlbom et al., 2001; Hammond et al., 2001; Huppenbauer et al., 2005; Pike, 2001; Ramsden et al., 2003), and in fact, many protective substances, including some that are known to be regulated by androgens, have already been shown to prevent motoneuron death following VRA. These include nitroarginine (an inhibitor of nitric oxide synthase; Wu and Li, 1993), brain-derived neurotrophic factor (Blits et al., 2004; Chai et al., 1999; Kishino et al., 1997; Novikov et al., 1995; Novikov et al., 1997; Novikova et al., 1997; Wu et al., 2003), glial cell line-derived neurotrophic factor (Blits et al., 2004; Li et al., 1995; Wu et al., 2003), IGF-I (Haninec et al., 2003), GM<sub>1</sub> ganglioside (Oliveira and Langone, 2000), T-588 (Ikeda et al., 2003), and Cerebrolysin (Haninec et al., 2003). It was therefore important to determine whether testosterone treatment prevented ipsilateral motoneuron death following VRA in the present experiment. The results indicate testosterone is completely ineffective in preventing motoneuron death following VRA (see Figure 5.2). This means that any potential beneficial effect of testosterone treatment on the morphology of the



contralateral motoneurons cannot be attributed to testosterone attenuating the motoneuron depletion induced by VRA.

### Morphometry

It has already been established that SNB motoneurons of untreated castrates undergo somal and dendritic atrophy (Araki et al., 1991; Breedlove and Arnold, 1981; Collins et al., 1992; Fargo and Sengelaub, 2004a; Forger et al., 1992; Jordan et al., 2002; Kurz et al., 1986; Sasaki and Arnold, 1991; Yang and Arnold, 2000a; Yang et al., 2004), and they are included in this experiment as a comparison group. Specifically, they offer evidence that my measurements are sensitive enough to detect changes in soma size and dendritic length.

### Somata

In the present experiment, avulsion of the ventral roots L5-S1 did *not* result in changes to the size of contralateral SNB somata, so there was no somal atrophy for the testosterone treatment to prevent (Figures 5.3 and 5.4). The somata of normal controls, VRA animals, and testosterone-treated VRA animals were very similar. Because castrated controls had significantly smaller somata, it is unlikely that the lack of a VRA effect is due to the assay being insufficiently sensitive to detect a difference. The lack of a VRA effect on contralateral somata stands in contrast to depletion experiments using saporin injection (Fargo and Sengelaub, 2004a, 2004b; Chapter 6). Why VRA failed to result in the atrophy of contralateral SNB somata is an important question, and I will address it below. Because VRA did not result in somal atrophy, I cannot reach a conclusion as to whether testosterone's neuroprotective effect following contralateral

motoneuron depletion generalizes to situations in which there is not an exogenous toxin introduced into the spinal cord.

### Dendrites

Group differences in dendritic length failed to reach significance. However, from a visual examination of the data (Figure 5.6) it appears that castrated controls had clearly shorter dendritic arbors than any other group, while the other groups had very similar arbors.<sup>4</sup> Additionally, previous studies have established that castration results in the marked atrophy of SNB dendrites (Fargo and Sengelaub, 2004a; Kurz et al., 1986; Sasaki and Arnold, 1991; Yang et al., 2004). In contrast, neither VRA nor testosterone treatment had any effect on dendritic length. As with soma size, this lack of an effect stands in contrast to depletion experiments using saporin injection (Fargo and Sengelaub, 2004a, 2004b; Chapter 6) in which depletion resulted in severe dendritic retraction. Why VRA failed to result in the atrophy of contralateral SNB dendrites is an important question, and I will address it below. Because VRA did not result in dendritic atrophy, I cannot reach a conclusion as to whether testosterone's neuroprotective effect following contralateral motoneuron depletion generalizes to situations in which there is not an exogenous toxin introduced into the spinal cord.

Analysis of the radial distribution of dendritic length revealed a significant interaction between group and location (Figure 5.7). Specifically, the dendritic length of VRA animals (regardless of testosterone treatment) is preferentially reduced in the most ventromedial portion of the spinal cord (240° to 300°). Interestingly, this happens to be

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<sup>4</sup> One might also speculate that VRA animals and testosterone-treated VRA animals had smaller dendritic arbors than normal controls, but that group differences were obscured by relatively large variances; however, the variances in these groups are not out of line with variances seen in previous experiments in which group differences in arbor per cell were statistically significant.

the portion of the spinal cord in which SNB motoneurons extend dendrites to connect with their contralateral counterparts. Perhaps the loss of contralateral SNB motoneurons results in a highly localized resculpting of the dendritic arbor, in which dendrites projecting to the now dead motoneurons are lost.<sup>5</sup>

The possibility that saporin injection could depress retrograde transport of HRP and produce spuriously low measures of dendritic length in the castrated controls is an important consideration. However, measures of dendritic extent would also be affected by depression of retrograde transport, and in the current experiment radial extent (Figure 5.8), rostral dendritic extent, and caudal dendritic extent were all unaffected by castration, making this unlikely.

#### Why doesn't VRA result in contralateral motoneuron atrophy?

The purpose of the present experiment was to test whether testosterone's neuroprotective effects on motoneuron morphology generalize to situations in which partial motoneuron depletion occurs in the absence of an exogenous toxin. To that end, I used unilateral ventral root avulsion to kill the majority of ipsilateral SNB motoneurons, and simultaneously treated some of the experimental animals with testosterone. Four weeks after VRA, more than 65% of ipsilateral SNB motoneurons were dead. This result accords very closely with the results of depletion experiments using intramuscular saporin injection (Fargo and Sengelaub, 2004a, 2004b; Chapter 6), so I was very surprised that neither overall dendritic length nor soma area was reduced in these animals. Not only does the lack of an effect of VRA on SNB morphology leave me unable to determine whether testosterone's benefits generalize, it also raises an

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<sup>5</sup> Note that in this case a highly localized resculpting might not necessarily be a pathological change, so the fact that treatment with testosterone does not prevent this does not necessarily argue against testosterone as a neurotherapeutic agent in this setup.

interesting question—*why* doesn't motoneuron depletion by ventral root avulsion result in contralateral motoneuron atrophy?

The obvious difference between saporin injection and VRA is that VRA does not introduce a toxic substance into the spinal cord. Motoneurons that have been disrupted by ventral root avulsion undergo apoptosis (Haninec et al., 2003; Hoang et al., 2003; Koliatsos et al., 1994), a form of cell death that does not result in either the spillage of the cytoplasm into the extracellular milieu or an inflammatory response. The remains of cells that have undergone apoptosis are simply cleared from the area. In contrast, while saporin may impinge on certain apoptotic pathways (Bergamaschi et al., 1996; Narayanan et al., 2005), its primary action is to inactivate ribosomes (Bergamaschi et al., 1996; Robertus and Monzingo, 2004; Stirpe, 2004). This leads to necrosis, a form cell death in which the cytoplasm is spilled into the extracellular space and which is typically followed by a strong local inflammatory response. These sequelae of necrosis might put stress on the surrounding tissues, including the contralateral motoneurons, thus resulting in contralateral motoneuron atrophy. Additionally, in this particular case, necrosis might result in the spilling of saporin into the extracellular space surrounding the surviving motoneurons. If this saporin remains in a biologically active state (Santanché et al., 1997), it could potentially enter the surviving contralateral motoneurons or other neurons or glia that express GM<sub>1</sub>. In fact, Jasmin and colleagues (Jasmin et al., 2000; Jasmin and Ohara, 2004) have introduced saporin into the spinal cord by intrathecal perfusion, and have found that this leads to extensive death of glia, and over time, motoneurons. So it is possible that the atrophy observed in saporin injected animals is a result of saporin acting

directly on those motoneurons, rather than being secondary to the death, *per se*, of their contralateral counterparts.

Another difference between saporin injection and ventral root avulsion is that VRA may take longer than saporin to induce significant amounts of cell death. Data from my previous work (Fargo and Sengelaub, 2004a) indicate that saporin leads to massive cell death within the first week, and the number of surviving cells don't change much between 1 and 4 weeks post injection. In contrast, VRA appears to lead to a slower, more progressive depletion of motoneurons (Wu, 1993). While Koliatsos et al. (1994) report seeing evidence of some cell death within 3 to 4 days following VRA, Hoang et al. (2003) report that it takes 1 week for 17% to die, 2 weeks for 54% to die, 4 weeks for 78% to die, and 6 weeks for 84% to die. In addition, Gu et al. (2004) report seeing progressive death of motoneurons as far out as 20 weeks after VRA. Given that the motoneuron depletion induced by VRA is slower and more progressive than that seen after saporin injection, it is reasonable to wonder whether I would have seen contralateral atrophy had I waited a few more weeks after VRA to examine morphology.<sup>6</sup> It is also possible that the slow, progressive nature of motoneuron loss following VRA allows the contralateral motoneurons to compensate for the loss, while the rapid nature of saporin-induced motoneuron loss overwhelms their capacity to do so.

Another possible difference is that while VRA kills motoneurons, saporin injected into the muscles may kill afferents as well as motoneurons. While this is purely speculative, it raises the interesting possibility that contralateral somal and dendritic atrophy, which are seen following saporin injection but not VRA, are induced by

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<sup>6</sup> It is also interesting to speculate about the localized dendritic retraction seen in root-avulsed animals between 240° and 300° (Figure 5.6); perhaps that is simply the first stage of a full-blown atrophy beginning to occur as more and more motoneurons die in the weeks following VRA.

deafferentation rather than by the death of motoneurons, *per se*. One simple way to test this hypothesis would be to perform dorsal root avulsions. If saporin-induced somal and dendritic atrophy arise from deafferentation, then dorsal root avulsions would be expected to cause similar atrophy while ventral root avulsions would not.

### Conclusions

The purpose of this experiment was to determine whether testosterone would have a protective effect on motoneuron morphology following contralateral motoneuron depletion by a method that does not introduce a toxin into the spinal cord. Ventral root avulsion was chosen because it is known to kill the vast majority of motoneurons ipsilateral to the injury. While VRA was successful in killing motoneurons unilaterally, I did not observe any contralateral motoneuron atrophy. Because of this, it is impossible to reach a conclusion as to the generalizability of testosterone's neuroprotective effects. Additional experiments need to be carried out to determine whether contralateral motoneurons would display atrophy at longer time points or following dorsal root avulsion. If they do, then testosterone treatment could be employed to see whether it is neuroprotective in that situation.

## Chapter 6.

Is Testosterone's Protective Effect on Surviving Motoneurons  
an Androgen Effect or an Estrogen Effect?

My previous studies (Fargo and Sengelaub, 2004a, 2004b) demonstrated that testosterone is neuroprotective following unilateral saporin injection. I have discussed this in terms of testosterone's role as an androgen. However, testosterone also acts on estrogenic systems by aromatization to estradiol. Additionally, both androgens and estrogens have protective qualities in the nervous system (Bialek et al., 2004; Henderson and Reynolds, 2002). It is therefore important to determine whether the effect of testosterone in protecting surviving motoneurons is an estrogen effect or an androgen effect.

The anatomy of the SNB/BC neuromuscular system has traditionally been thought of as primarily androgen-dependent. SNB motoneurons contain high levels of both androgen receptors (Breedlove and Arnold, 1980) and 5 $\alpha$ -reductase (the enzyme that converts testosterone into the powerful androgen dihydrotestosterone; Pozzi et al., 2003), as well as steroid receptor coactivators (O'Bryant and Jordan, 2005; Ranson et al., 2003), all of which suggest that these motoneurons are direct androgen targets. Additionally, androgens maintain a wide variety of SNB motoneuron characteristics, including soma size (Breedlove and Arnold, 1981) and dendritic length (Kurz et al., 1986), the number and size of synapses (Leedy et al., 1987; Matsumoto, 2005; Matsumoto et al., 1988b) and gap junction plaques (Matsumoto et al., 1988a), and the percentage of membrane contacted by glia (Leedy et al., 1987). Moreover, the SNB target musculature contains high numbers of androgen receptors (Monks et al., 2004); and androgens maintain a variety of characteristics of these muscles, including weight (Wainman and Shipounoff, 1941), fiber size (Venable, 1966), neuromuscular junction size (Balice-Gordon et al., 1990; Bleisch and Harrelson, 1989), acetylcholine receptor number (Bleisch and



Harrelson, 1989; Bleisch et al., 1982), muscle excitability (Foster and Sengelaub, 2004b), and the number of functional calcium channels at the neuromuscular junction (Nudler et al., 2005).

Androgens also regulate several important biochemicals in the adult rat SNB/BC neuromuscular system. For example, levels of immunoreactivity for the ciliary neurotrophic factor receptor  $\alpha$  are androgen-dependent in SNB motoneurons (Forger et al., 1998), as are levels of mRNA expression for the major cytoskeletal elements  $\beta$ -actin (Matsumoto et al., 1992) and  $\beta$ -tubulin (Matsumoto et al., 1993). Additionally, androgen manipulation affects levels of gap junction mRNA expression (Matsumoto et al., 1991; Matsumoto et al., 1992), N-cadherin immunoreactivity (Monks et al., 2001), calcitonin gene-related peptide immunoreactivity (Popper and Micevych, 1989) and mRNA expression (Popper and Micevych, 1990), androgen receptor immunoreactivity (Matsumoto et al., 1996), and BCL-2 immunoreactivity (Zup and Forger, 2002) in SNB motoneurons.

However, the SNB/BC neuromuscular system is not regulated solely by androgens. The target muscles of the SNB also contain an estrogen-binding protein (presumably an estrogen receptor; Dubé et al., 1976), and estradiol plays a powerful role in this system. For example, the reflexes of this system are maintained by estradiol following castration (O'Hanlon et al., 1981). In addition, Holmes and Sachs (1992) found that estradiol was as effective as testosterone in maintaining EMG activity of the BC during copulation. Estradiol-treated castrates displayed spontaneous erections during copulatory testing, and produced *in copula* reflexive BC EMG activity similar to that of intact males and testosterone-treated castrates. Estradiol also maintains the motor

excitability of the SNB/BC in response to nerve stimulation (Fargo et al., 2003; Foster and Sengelaub, 2004b). Finally, estradiol is necessary for the development of normal motoneuron morphology in the SNB (Burke et al., 1999; Burke et al., 1997; Goldstein and Sengelaub, 1994; Hebbeler et al., 2001).

Given this system's responsiveness to both androgens and estrogens, and given that testosterone can act as an androgen or be metabolized into either an androgen or an estrogen (both of which exert neuroprotective effects in the CNS), it is important to determine whether the effect of testosterone in maintaining SNB motoneuron morphology following saporin injection is an androgen effect or an estrogen effect. The purpose of the present experiment is to make this discrimination by injecting animals with saporin, then treating them with either estradiol or the non-aromatizable androgen dihydrotestosterone.

## METHODS AND DESIGN

Adult male Sprague Dawley rats were used. Saporin injections were made into the BC and LA muscles according to the method described in Chapter 2 (saporin only  $n = 8$ ). Coincident with saporin injection, some animals were also given replacement hormones—testosterone, dihydrotestosterone, or estradiol. Testosterone implants were made and administered according the methods described in Chapter 2 (saporin and testosterone treatment  $n = 8$ ). Dihydrotestosterone (DHT) implants were made in the same fashion as testosterone implants with the exception that the diffusion surface of the implant was 30 mm long instead of 45 mm long. This length was chosen because DHT implants of this size and even smaller have been used with robust results in previously published studies (Forger et al., 1992; Meisel et al., 1984; Monks et al., 2001). In

addition, this laboratory has previously used 30-mm DHT implants to successfully maintain dendritic length following castration (unpublished observations), further increasing my confidence that this length would dispense a sufficient amount of DHT to demonstrate an effect. DHT was chosen because it exerts its effects through the androgen receptor, and it cannot be aromatized into estradiol; therefore, if DHT has an effect, it will not be attributable to conversion to estradiol (saporin and DHT treatment  $n = 8$ ).

In contrast to androgen treatment, estrogen treatment was given in the form of subcutaneous injections of hormone dissolved in sesame oil. Estradiol-treated animals received daily injections beginning on the day of saporin injection and castration. Each injection consisted of 300  $\mu\text{g}$  estradiol benzoate (Steraloids, Inc.; Wilton, NH) dissolved in 0.15 ml of sesame oil (saporin and estradiol treatment  $n = 7$ ). This dose was chosen because I have previously used it to successfully maintain electrophysiological activity at normal levels in castrated males (Fargo et al., 2003; also see Holmes and Sachs, 1992). Oil-based injections were chosen over Silastic implants because oil-based injections have previously been used to successfully mediate SNB dendritic morphology in rats (Burke et al., 1997; Goldstein and Sengelaub, 1994; Hebbeler and Sengelaub, 2003); in contrast, I am unaware of any previous studies demonstrating an effect of Silastic estradiol implants on motoneuron morphology. Estradiol benzoate is a physiologically active ester of estradiol. When administering hormones in oil, hormone esters are typically used in place of standard hormones; because they are strongly hydrophobic they stay dissolved in the oil vehicle longer and thus provide a more steady level of hormone throughout the day (Smith et al., 1977). A group of untreated intact normal males served as a control group ( $n = 11$ ).

It should be noted that no animals were castrated in this experiment. This is a departure from previous experiments, in which hormone-treated animals were castrated. As discussed in Chapter 2, the purpose of castration in those animals was to improve experimenter control over hormone levels by eliminating one source of inter-individual variation in hormone levels, namely, the amount of hormones produced by each animal's gonads. However, the purpose of the present experiment was to determine whether treatment with testosterone or its immediate metabolites, DHT and estradiol, would prevent saporin-induced dendritic atrophy. Previous experiments (unpublished data from this laboratory) have demonstrated that estradiol treatment is insufficient to prevent or reverse castration-induced dendritic retraction. For this reason, if the estradiol-treated animals in this experiment had been castrated, it would have been impossible to determine whether any potential dendritic atrophy was caused by the castration or by an inability of estradiol to prevent *saporin*-induced atrophy. In addition, the fact that this experimental design does not include castration allows me to determine whether androgen treatment is effective in preventing saporin-induced dendritic atrophy in gonadally intact animals.

Four weeks after saporin injection and hormone-treatment onset, I injected HRP into the contralateral BC muscle according to the method described in Chapter 2. After two additional days to allow for optimal HRP transport, animals were killed and the perineal musculature removed and weighed. Lumbosacral spinal cords were removed and processed for motoneuron counts and morphometry according to the methods described in Chapter 2.

## RESULTS

### Muscle Weights

Unilateral injection of saporin into the BC/LA muscle complex resulted in marked atrophy of the injected musculature [ $0.444 \pm 0.016$  g for saporin-injected animals compared to  $0.606 \pm 0.030$  g for normal controls, LSD,  $p < .001$ ; overall test for the effect of group on right side muscle weight  $F(4, 30) = 49.47$ ,  $p < .001$ ; see Figure 6.1], and hormone treatment did not prevent this (right side muscle weights were  $0.514 \pm 0.021$  g for saporin-injected animals treated with testosterone,  $0.189 \pm 0.013$  g for saporin-injected animals treated with estradiol, and  $0.424 \pm 0.024$  g for saporin-injected animals treated with DHT; compared to normal controls, LSD,  $ps < .01$ ). However, while none of the various hormone treatments completely prevented saporin-induced reductions in muscle weight, they did have differential effects: testosterone increased muscle weight to some extent while estradiol further decreased it (compared to untreated saporin-injected animals; LSD,  $ps < .05$ ), and DHT had no effect (compared to untreated saporin-injected animals; LSD,  $ns$ ). Notably, the effect of saporin injection on BC/LA weight was specific to the injected side of the muscle complex [left side muscle weights were  $0.603 \pm 0.017$  g for saporin-injected animals and  $0.612 \pm 0.030$  g for normal controls, LSD,  $ns$ ; overall test for the effect of group on left side muscle weight  $F(4, 30) = 50.59$ ,  $p < .001$ ; see Figure 6.1]. The various hormone treatments had differential effects on left side muscle weights also: testosterone increased muscle weight ( $0.727 \pm 0.039$  g for saporin-injected animals treated with testosterone; compared to untreated saporin-injected animals, LSD,  $p < .01$ ) while estradiol decreased it markedly and DHT decreased it a small amount ( $0.243 \pm 0.016$  g for saporin-injected animals treated with estradiol, and

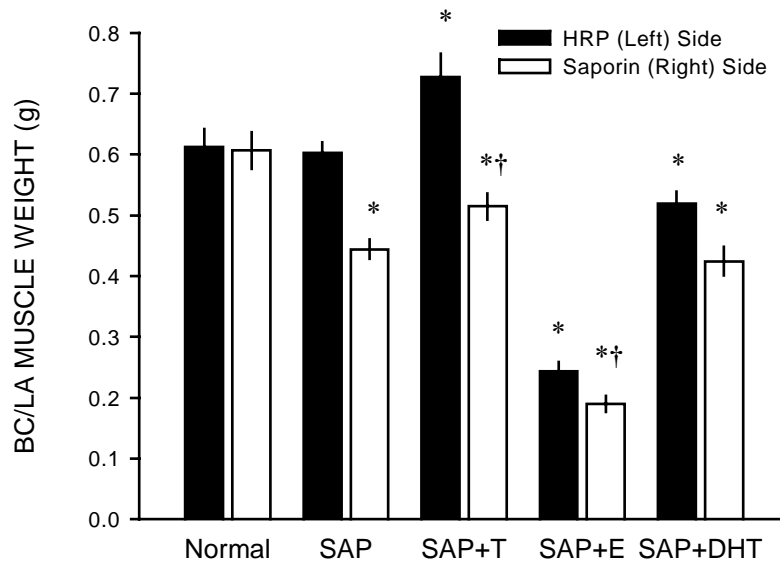


FIGURE 6.1

BC/LA muscle weights for normal controls, untreated saporin-injected animals (SAP), saporin-injected animals treated with testosterone (SAP+T), saporin-injected animals treated with estradiol (SAP+E), and saporin-injected animals treated with the non-aromatizable androgen dihydrotestosterone (SAP+DHT). \* indicates significantly different from normal controls,  $p < .05$ . † indicates significantly different from untreated saporin-injected animals,  $p < .05$ . Bar heights represent means  $\pm$  SEM. Open bars: Open bars represent the saporin-injected side of the muscle complex. Saporin injection decreased muscle weight on the injected side. Steroid treatments had differential effects. Testosterone increased muscle weight, but only partially attenuated the effect of saporin injection. Estradiol actually decreased muscle weight even more than saporin injection alone. DHT neither increased nor decreased muscle weight compared to saporin injection alone. Filled bars: Filled bars represent the HRP-injected side of the muscle complex. Saporin injection into the contralateral side had no effect on this side of the muscle. Steroid treatments had differential effects. Testosterone increased muscle weight above normal. In contrast, both estradiol and DHT reduced muscle weight, with estradiol having a much larger effect than DHT.

Note: For normal controls, the open bars represent the right (non-injected) side and the filled bars represent the left (HRP-injected) side of the BC/LA muscle complex.

0.520 ± 0.019 g for saporin-injected animals treated with DHT; compared to untreated saporin-injected animals, LSD,  $p < .05$ ).

### Cell Counts

The number of SNB motoneurons in normal controls was well within the normal range (108.80 ± 11.34). Injection of saporin into the BC/LA muscle complex resulted in the death of over 60% of ipsilateral SNB motoneurons [42.00 ± 5.07 remaining motoneurons for saporin-injected animals; compared to normal controls, LSD,  $p < .001$ ; overall test for the effect of group on motoneuron number  $F(4, 25) = 17.25$ ,  $p < .001$ ; see Figure 6.2], and hormone treatment did not prevent this (34.67 ± 6.42 remaining motoneurons for saporin-injected animals treated with testosterone, 32.00 ± 4.73 remaining motoneurons for saporin-injected animals treated with estradiol, and 33.33 ± 8.98 remaining motoneurons for saporin-injected animals treated with DHT; compared to normal controls, LSD,  $p < .001$ ). Importantly, SNB motoneuron death induced by saporin injection was specific to the ipsilateral side: on the contralateral side, the average number of remaining motoneurons was 92.27 ± 5.19, and this did not differ between groups [ $F(4, 25) = 0.08$ ,  $ns$ ]

### Morphometry

#### Somata

Somata were traced for an average of 22.50 ± 0.73 HRP-labeled motoneurons per animal, and this did not differ between groups [ $F(4, 23) = 1.35$ ,  $ns$ ]. Injection of saporin into the BC/LA muscle complex resulted in the atrophy of contralateral SNB motoneurons. Soma areas decreased by approximately 20% [875.44 ± 44.69  $\mu\text{m}^2$  for saporin-injected animals compared to 1068.71 ± 57.88  $\mu\text{m}^2$  for normal controls, LSD,  $p <$

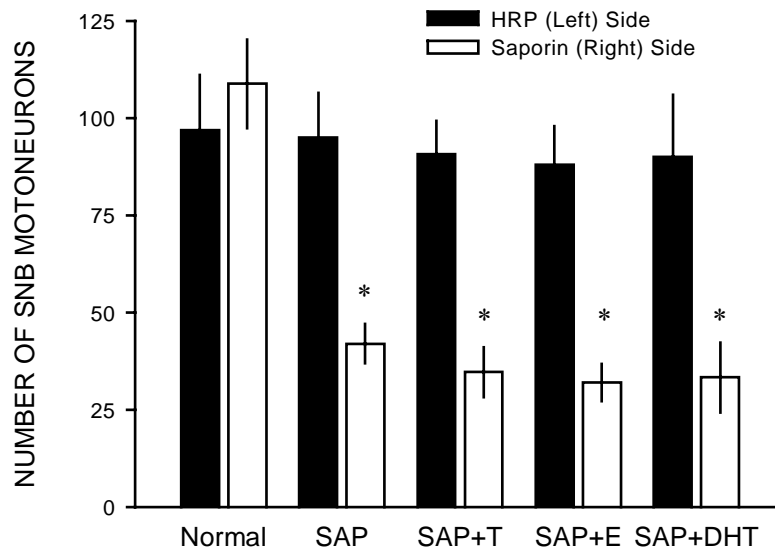


FIGURE 6.2

Numbers of thionin-stained SNB motoneurons for normal controls, untreated saporin-injected animals (SAP), saporin-injected animals treated with testosterone (SAP+T), saporin-injected animals treated with estradiol (SAP+E), and saporin-injected animals treated with the non-aromatizable androgen dihydrotestosterone (SAP+DHT), for both the saporin-injected side (open bars) and the HRP-injected side (filled bars). Saporin killed more than 60% of the ipsilateral SNB motoneurons, regardless of hormone status, and did not kill contralateral motoneurons. \* indicates significantly different from normal controls,  $p < .001$ . Bar heights represent means  $\pm$  SEM.

Note: For normal controls, the open bars represent the right half and the filled bars represent the left half of the SNB.



.05; overall test for the effect of group on soma area  $F(4, 23) = 4.46, p < .01$ ; see Figure 6.3]. However, treatment with androgens prevented this atrophy ( $921.44 \pm 48.09 \mu\text{m}^2$  for saporin-injected animals treated with testosterone, and  $934.41 \pm 50.80 \mu\text{m}^2$  for saporin-injected animals treated with DHT; compared to normal controls, LSDs, *ns*). In contrast, treatment with estradiol was completely ineffective in protecting contralateral SNB motoneurons from saporin-induced reductions in soma area ( $750.49 \pm 58.06 \mu\text{m}^2$  for saporin-injected animals treated with estradiol; compared to normal controls, LSD,  $p < .001$ )

### Dendrites

Injection of HRP into the left BC successfully labeled ipsilateral SNB motoneurons in a manner consistent with previous studies (Fargo and Sengelau, 2004a, 2004b; Kurz et al., 1991; Kurz et al., 1986). SNB motoneurons displayed their characteristic multipolar morphologies (Kurz et al., 1986; Sasaki and Arnold, 1991), with dendritic arbors projecting ventrolaterally, dorsomedially, and across the midline into the area of the contralateral SNB (Figure 6.4). An average of  $50.57.10 (\pm 3.96)$  motoneurons per animal were labeled with HRP.

Injection of saporin into the BC/LA muscle complex resulted in the marked atrophy of contralateral SNB motoneuron dendrites. Dendritic length decreased by more than 65% ( $2589.31 \pm 399.26 \mu\text{m}$  for saporin-injected animals compared to  $7694.93 \pm 1728.55 \mu\text{m}$  for normal controls, LSD,  $p < .01$ ; overall test for the effect of group in arbor per cell  $F(4, 23) = 4.66, p < .01$ ; see Figure 6.5]. However, treatment with androgens prevented this dendritic atrophy ( $5234.49 \pm 1195.14 \mu\text{m}$  for saporin-injected animals treated with testosterone, and  $7181.43 \pm 1435.71 \mu\text{m}$  for saporin-injected animals treated

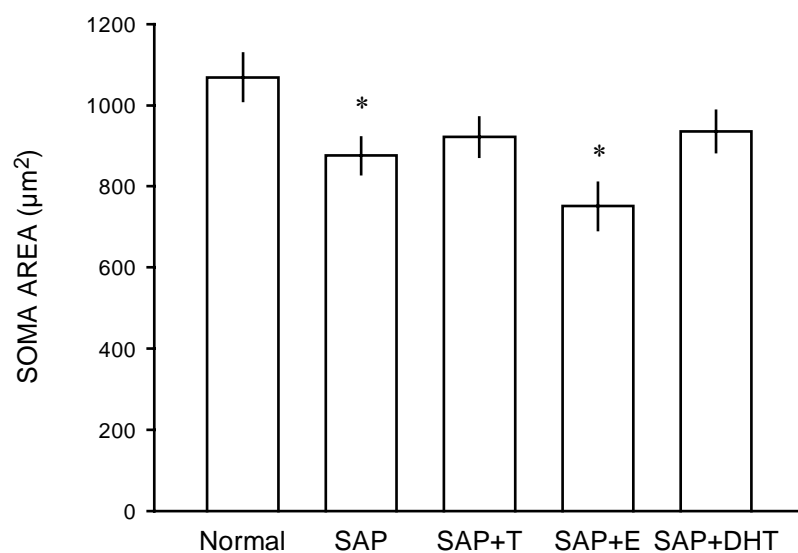


FIGURE 6.3

Cross-sectional soma areas of SNB motoneurons for normal controls, untreated saporin-injected animals (SAP), saporin-injected animals treated with testosterone (SAP+T), saporin-injected animals treated with estradiol (SAP+E), and saporin-injected animals treated with the non-aromatizable androgen dihydrotestosterone (SAP+DHT). Saporin injection decreased contralateral soma size by approximately 20%. Treatment with either testosterone or DHT prevented this decrease, but treatment with estradiol was completely ineffective. \* indicates significantly different from normal controls,  $p < .05$ . Bar heights represent means  $\pm$  SEM.

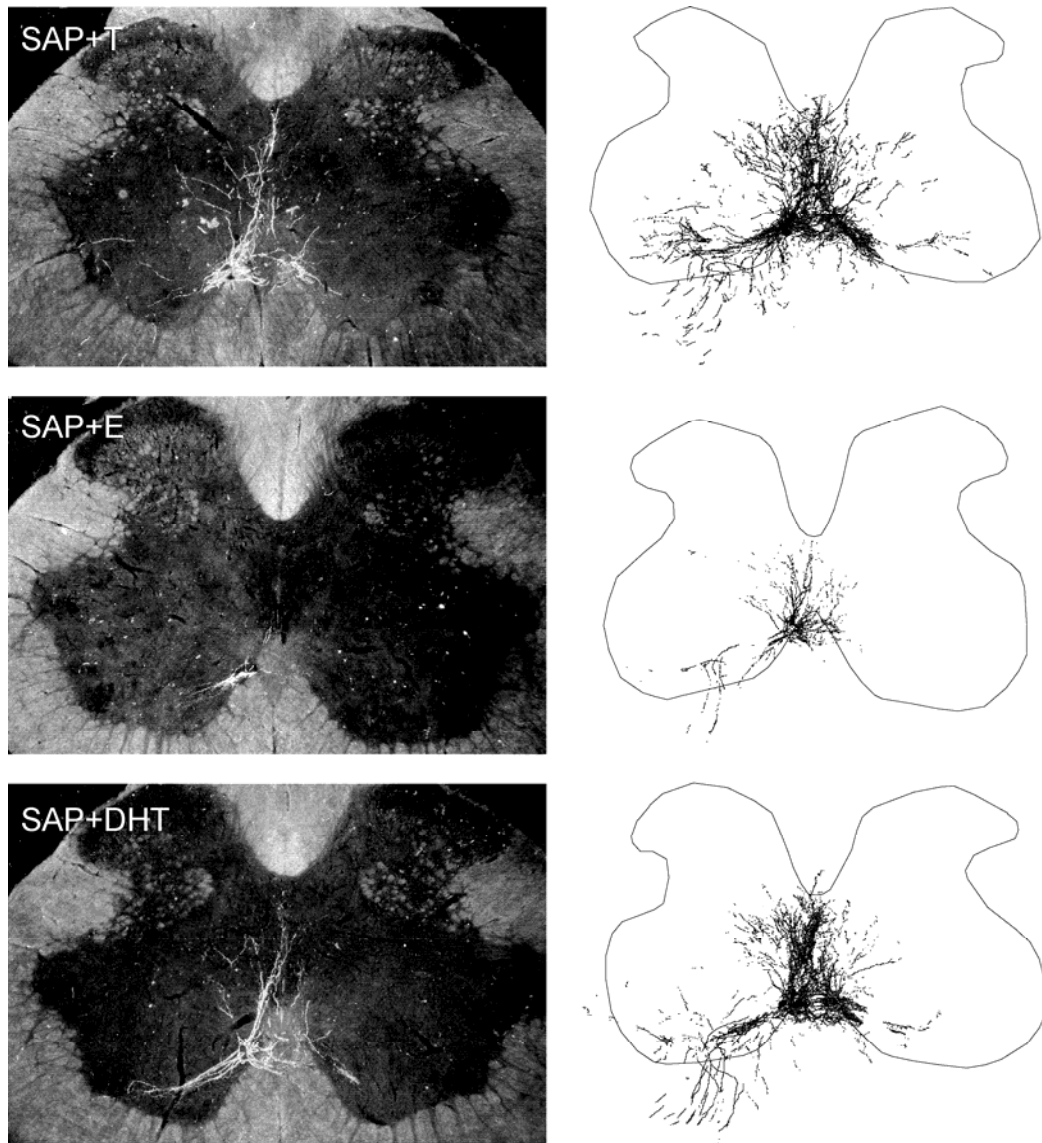


FIGURE 6.4

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

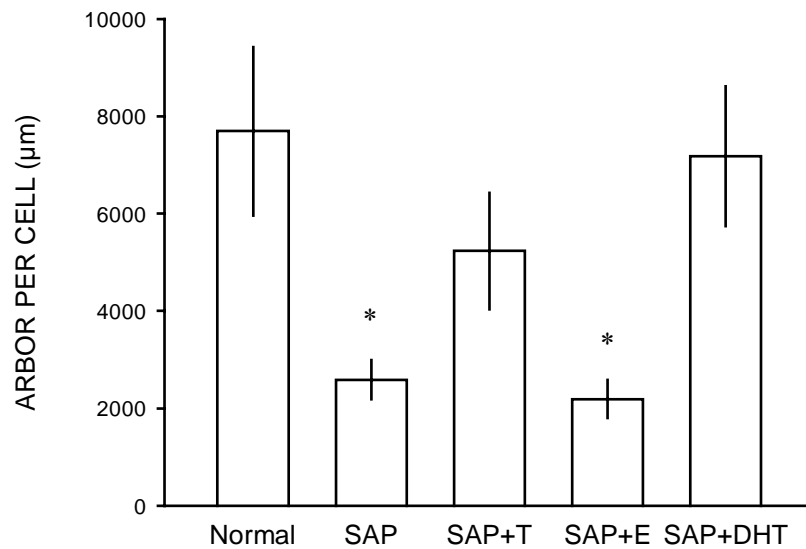


FIGURE 6.5

Dendritic lengths of SNB motoneurons of normal controls, untreated saporin-injected animals (SAP), saporin-injected animals treated with testosterone (SAP+T), saporin-injected animals treated with estradiol (SAP+E), and saporin-injected animals treated with the non-aromatizable androgen dihydrotestosterone (SAP+DHT). Saporin injection caused a greater than 65% reduction in dendritic length. Treatment with either testosterone or the non-aromatizable androgen DHT prevented this dendritic atrophy. In contrast, treatment with estradiol was completely ineffective. \* indicates significantly different from normal controls,  $p < .01$ . Bar heights represent means  $\pm$  SEM.

with DHT; compared to normal controls, LSDs, *ns*]. In contrast, treatment with estradiol was completely ineffective in protecting contralateral SNB motoneurons from saporin-induced dendritic atrophy ( $2193.08 \pm 384.16 \mu\text{m}$  for saporin-injected animals treated with estradiol; compared to normal controls, LSD,  $p < .01$ ).

Dendritic length was nonuniform across radial bins, and repeated-measures ANOVA revealed a significant effect of radial location [ $F(11, 253) = 111.39, p < .001$ ; see Figure 6.6]. Consistent with the results of the arbor per cell analysis, there was also a significant effect of group [ $F(4, 23) = 4.75, p < .01$ ]. Saporin injection led to reductions in arbor length of approximately 91% from  $0^\circ$  to  $30^\circ$ , 90% from  $30^\circ$  to  $60^\circ$ , 93% from  $60^\circ$  to  $90^\circ$ , 94% from  $90^\circ$  to  $120^\circ$ , 90% from  $120^\circ$  to  $150^\circ$ , 87% from  $150^\circ$  to  $180^\circ$ , 79% from  $180^\circ$  to  $210^\circ$ , 30% from  $210^\circ$  to  $240^\circ$ , 44% from  $240^\circ$  to  $270^\circ$ , 79% from  $270^\circ$  to  $300^\circ$ , 88% from  $300^\circ$  to  $330^\circ$ , and 93% from  $330^\circ$  to  $360^\circ$ . Treatment with either testosterone or the non-aromatizable androgen DHT blocked these reductions, but treatment with estradiol did not (see Figure 6.6).

Dendritic extent was nonuniform across radial bins, and repeated-measures ANOVA revealed a significant effect of radial location [ $F(11, 253) = 100.81, p < .001$ ]. There was also a significant effect of group [ $F(4, 23) = 7.90, p < .001$ ]. Radial dendritic extent was depressed in both untreated saporin-injected animals and in saporin-injected animals treated with estradiol (compared to normal controls; LSD,  $ps < .05$ ). Only treatment with DHT completely protected SNB motoneurons from saporin-induced loss of radial dendritic extent (compared to normal controls; LSD, *ns*). While the radial dendritic extent of testosterone-treated saporin-injected animals was still significantly smaller than that of normal controls (LSD,  $p < .05$ ), an examination of the data makes it

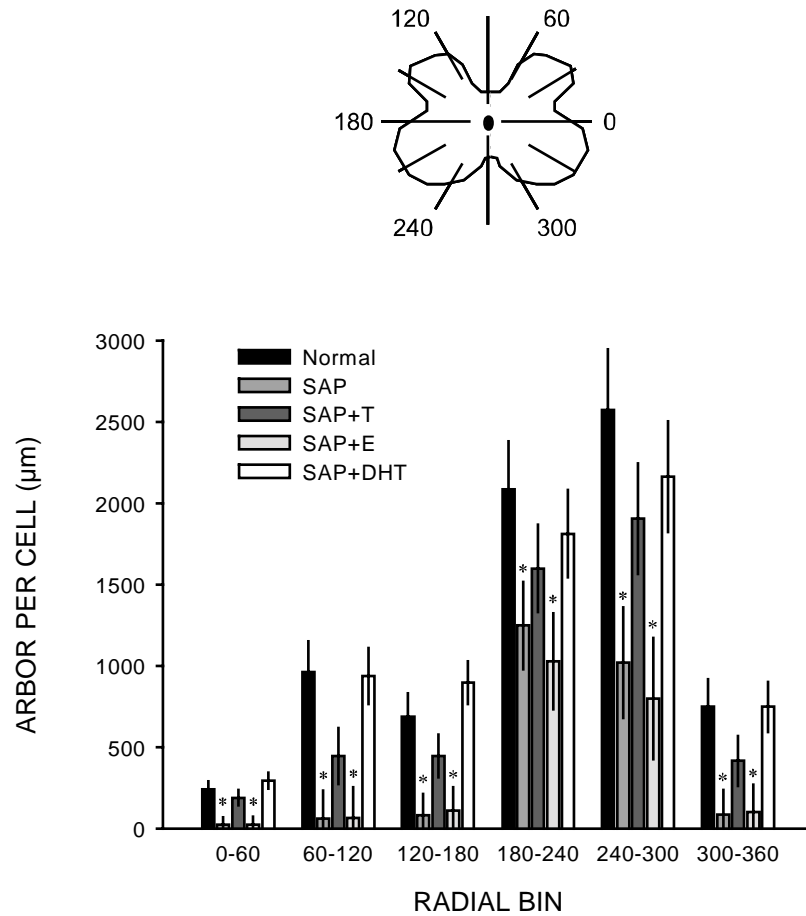


FIGURE 6.6

Top: Drawing of spinal gray matter divided into radial sectors for measure of SNB dendritic distribution. Bottom: Length per radial bin of SNB dendrites in normal controls, untreated saporin-injected animals (SAP), saporin-injected animals treated with testosterone (SAP+T), saporin-injected animals treated with estradiol (SAP+E), and saporin-injected animals treated with the non-aromatizable androgen dihydrotestosterone (SAP+DHT). For graphic purposes, dendritic length measures have been collapsed into 6 bins of 60° each. SNB motoneuron dendritic arbors display a non-uniform distribution, with the majority of the arbor located between 180° and 300°. Saporin injection and castration each reduced dendritic length in every bin. Treatment with testosterone or DHT effectively blocked this reduction, but treatment with estradiol did not. \* indicates significantly different from normal controls,  $p < .01$ . Bar heights represent means  $\pm$  SEM.

clear that testosterone treatment partially attenuated the effects of saporin injection in every radial bin (see Figure 6.7). There was no significant interaction between radial location and group [ $F(44, 253) = 1.16, ns$ ].

Rostrocaudal dendritic extent also varied by group [ $F(4, 23) = 3.30, p < .05$ ; see Figure 6.8]. However, post hoc comparisons revealed that no group differed significantly from normal controls ( $3008.00 \pm 259.97 \mu\text{m}$  for normal controls,  $2586.67 \pm 167.39 \mu\text{m}$  for untreated saporin-injected animals,  $3253.33 \pm 114.39 \mu\text{m}$  for saporin-injected animals treated with testosterone,  $2624.00 \pm 129.98 \mu\text{m}$  for saporin-injected animals treated with estradiol, and  $3200.00 \pm 184.75 \mu\text{m}$  for saporin-injected animals treated with DHT; LSDs, *ns*).

## DISCUSSION

### Muscle Weights

Given the hypothesis that testosterone might exert its beneficial effects on motoneuron morphology by acting through the muscle, the muscle weight results are of particular interest (Figure 6.1). The first thing that should be noted is that saporin injection decreased the weight of the injected side of the muscle complex in every group. Second, in the untreated saporin-injected animals, saporin injection had no effect on the weight of the contralateral muscles. These results provide a verification that saporin injections occurred as planned. Specifically, they increase our confidence that saporin did not diffuse across the midline in the periphery in any significant amount.

Testosterone and its metabolites had very different effects on muscle weights (Figure 6.1). Testosterone was the most trophic of the three—it increased the weight of the non-injected muscle above normal, and partially ameliorated the atrophy induced by

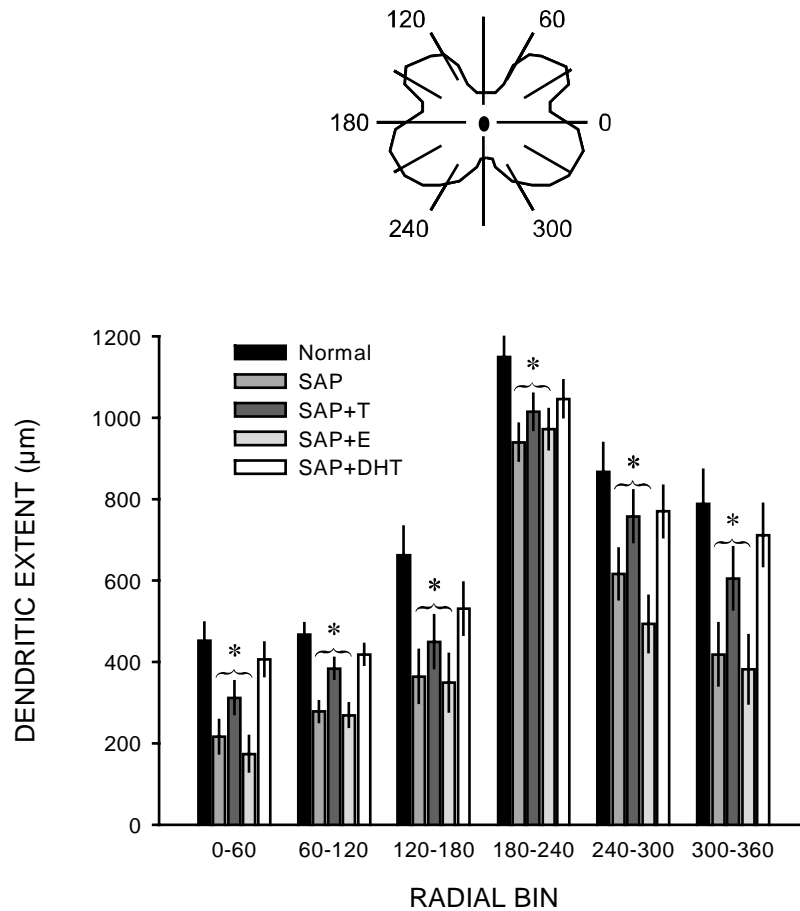


FIGURE 6.7

Top: Drawing of spinal gray matter divided into radial sectors for measure of SNB radial dendritic extent. Bottom: Radial extents of SNB dendrites in normal controls, untreated saporin-injected animals (SAP), saporin-injected animals treated with testosterone (SAP+T), saporin-injected animals treated with estradiol (SAP+E), and saporin-injected animals treated with the non-aromatizable androgen dihydrotestosterone (SAP+DHT). For graphic purposes, dendritic extent measures have been collapsed into 6 bins of 60° each. Saporin injection depressed SNB dendritic extent across radial bins. Treatment with DHT completely prevented this. Testosterone appeared to partially prevent the loss of radial dendritic extent (but this was not statistically significant; see text). Estradiol was completely ineffective in preventing decreases in radial dendritic extent. \* indicates significantly different from normal controls,  $p < .05$ . Bar heights represent means  $\pm$  SEM.



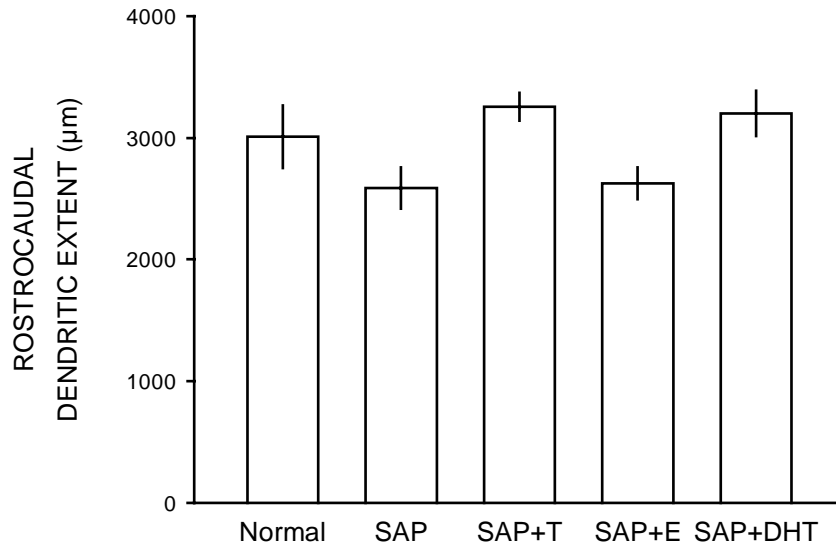


FIGURE 6.8

Rostrocaudal extents of SNB dendrites in normal controls, untreated saporin-injected animals (SAP), saporin-injected animals treated with testosterone (SAP+T), saporin-injected animals treated with estradiol (SAP+E), and saporin-injected animals treated with the non-aromatizable androgen dihydrotestosterone (SAP+DHT). While an ANOVA revealed a main effect of group on rostrocaudal dendritic extent, no group differed significantly from normal controls. Bar heights represent means  $\pm$  SEM.

saporin in the injected muscle. In contrast, DHT had no beneficial effect on the weight of the saporin injected muscles, and even resulted in a small but significant *loss* of weight in the non-injected muscles. Given that DHT is an androgen and an anabolic steroid, it would seem unusual for it to actually cause muscle atrophy. In fact, it has previously been reported that DHT maintains BC/LA muscle weight in castrated animals over at least the first 10 days post-castration (Foster and Sengelaub, 2004b). However, the muscle weight data from Forger et al. (1992) and unpublished data from our laboratory independently confirm that BC and LA muscle weights are better maintained by testosterone than by DHT over longer periods. It is therefore likely that DHT treatment decreased muscle weight in these animals by depressing testosterone levels via a negative feedback loop, inhibiting the HPG axis. Finally, estradiol treatment dramatically reduced BC/LA muscle weights. Again, this is probably secondary to a depression of testosterone levels via negative feedback. The marked loss of muscle weight in the estradiol-treated animals is especially interesting in light of the fact that estradiol treatment has been shown to protect electrophysiological activity of the BC and LA by acting on peripheral structures (Fargo et al., 2003; Foster and Sengelaub, 2004b). It therefore seems that estradiol acts on some peripheral component of the SNB system that is independent of muscle weight.

### Cell Counts

Unilateral saporin injection resulted in the death of over 60% of ipsilateral motoneurons (Figure 6.2). Furthermore, saporin injection resulted in a laterally specific depletion of SNB motoneurons—motoneuron number contralateral to saporin injection did not differ from normal. As previously mentioned, in some model preparations, steroid

hormones prevent injury-induced neuron death. It was therefore important to establish that saporin injection killed approximately the same number of motoneurons in the untreated and steroid-treated groups. As can be clearly seen in Figure 6.2, saporin injection was equally effective in killing motoneurons in all saporin-injected groups, regardless of hormone treatment. This means that any potential beneficial effects of steroid treatment on contralateral motoneuron morphology cannot be explained simply by an attenuation of saporin's ability to kill ipsilateral motoneurons.

### Morphometry

The number of motoneurons labeled with HRP did not differ between groups. It is therefore unlikely that differences in measures of motoneuron morphology between groups were affected by potential differences in the number of motoneurons labeled with HRP.

### Somata

Saporin injection resulted in the somata of contralateral motoneurons losing about 20% of their cross-sectional area (Figure 6.3), consistent with the results of my previous studies (Fargo and Sengelau, 2004a, 2004b). Treatment with either testosterone or DHT prevented this atrophy, but treatment with estradiol did not. These data indicate that the neuroprotective effect of testosterone treatment on soma size in this model is an androgenic effect.

### Dendrites

The arbor per cell results mirror the soma size results (Figure 6.5). Saporin injection resulted in the loss of more than 65% of the dendritic arbor of contralateral motoneurons. Treatment with either testosterone or DHT prevented this, but treatment

with estradiol did not. Analysis of the radial distribution of dendritic length revealed that saporin injection resulted in the loss of dendritic length in every radial bin; furthermore, treatment with either testosterone or the non-aromatizable androgen DHT, but not estradiol, prevented these decreases in every radial bin (Figure 6.6). These data indicate that the neuroprotective effect of testosterone treatment on dendritic length in this model is an androgenic effect.

Dendritic extent displayed a pattern similar to overall dendritic length. Saporin injection decreased radial dendritic extent across all radial bins (Figure 6.7). Estradiol treatment was completely ineffective in preventing this. However DHT treatment *did* prevent this loss, and an examination of Figure 6.7 suggests that testosterone treatment was also partially effective, even though testosterone-treated animals were still significantly different from normal controls. These data suggest that androgen treatment exerts a protective effect on dendritic extent as well as dendritic length, while estradiol is ineffective by all of these measures.

#### Is steroid neuroprotection exclusively androgenic in this system?

Because both testosterone and DHT were neuroprotective, while estradiol was not, the present results suggest that the neuroprotective effects of steroid treatment following saporin injection in this system are androgenic in nature. This is consistent with what is already known about adult morphological plasticity in the SNB. For example, following castration both testosterone and DHT are capable of maintaining SNB soma size (Forger et al., 1992; Verhovshek et al., 2000) and dendritic length (Verhovshek et al., 2000). In contrast, estradiol replacement during adulthood fails to prevent castration-induced atrophy of SNB motoneuron somata (Forger et al., 1992; Verhovshek et al.,

2000) or dendrites (Verhovshek et al., 2000). Similarly, blockade of estradiol synthesis in intact adult males with the aromatase inhibitor fadrozole has no effect on SNB motoneuron morphology (Verhovshek et al., 2000). Furthermore, SNB motoneurons accumulate androgens, but not estradiol (Breedlove and Arnold, 1980; Breedlove and Arnold, 1983), and express high levels of 5 $\alpha$ -reductase (Pozzi et al., 2003), the enzyme that converts testosterone into DHT.

However, estrogens have well-known trophic and neuroprotective effects (Cooke and Woolley, 2005; Garcia-Segura et al., 2001; Woolley and Cohen, 2002), and I think that it is still premature to completely rule out a role for estrogens in neuroprotection in the SNB. There are at least three reasons for this. First, the current experiment only tested the efficacy of estradiol, and there are of course other estrogens (such as estrone or estriol) that could potentially have neuroprotective effects. Second, although DHT is best known as a high-affinity ligand of the androgen receptor, recent reports from Robert Handa and colleagues indicate that DHT can be metabolized into steroids that act primarily via estrogen receptors (Lund et al., 2004; Pak et al., 2005). Finally, potential confounds exist in the design of the present experiment. Specifically, the dosage and route of administration for the androgens was different than for estradiol. While the amount of steroid in each group was chosen based on its efficacy in previous experiments, only testosterone had previously been used to prevent saporin-induced motoneuron atrophy, so I can't really be sure that estradiol wouldn't have been effective in this experiment had I used a higher dose. It is also possible that route of administration has something to do with steroid effectiveness in this preparation. In fact, one hypothesis as to why testosterone treatment is effective even though the implants used do not

produce supraphysiological levels of testosterone is that the implants eliminate natural diurnal fluctuations in serum testosterone levels. While estradiol levels do not fluctuate to the same extent as testosterone levels, and estradiol benzoate was used specifically to prolong exposure to estradiol throughout the day, it is highly doubtful that estradiol-treated animals received hormone at the same constant rates as the androgen-treated animals. In fact, it is known that steroid esters in oil cause a spike in plasma hormone levels shortly after each daily injection (Smith et al., 1977).

### Conclusions

The results of this experiment indicate that testosterone's neuroprotective effect following saporin injection is *not* mediated by its conversion to estradiol. Additionally, these data refute the hypothesis that testosterone's protective effect on motoneuron morphology is mediated by its effects on the weight of the musculature, because DHT also is protective of morphology, even though the muscles of DHT-treated animals were actually smaller than those of normal controls. In summary, then, testosterone exerts its neuroprotective effect on SNB morphology by acting as an androgen, and doing so independently of its effects on muscle weight.

## Chapter 7.

### General Discussion

The purpose of this dissertation was to further explore the neuroprotective and neurotherapeutic effects of testosterone treatment following the partial loss of motoneurons. To that end, I performed experiments aimed at answering several important questions raised by my previous studies on this topic (see Chapter 1 for a review of my previous work). In this final chapter of the thesis I summarize the primary findings of my dissertation research, relate them to each other and to my previous studies, discuss broadly the significance of this work, and make proposals for future research.

### Summary of Primary Findings

Cell count data revealed that experimental manipulations used to deplete motoneuron populations were very effective. Unilateral saporin injection into the BC muscle alone killed approximately 40% of ipsilateral SNB motoneurons (Figure 3.2); and unilateral saporin injection into both the BC and LA muscles killed more than 60% of ipsilateral SNB motoneurons while leaving contralateral motoneuron numbers unchanged (Figure 6.3). These results are consistent with my previous studies (Fargo and Sengelaub, 2004a, 2004b), and engender confidence that saporin injection was successful in killing only motoneurons projecting to the injected musculature. Similarly, unilateral ventral root avulsion killed more than 65% of ipsilateral SNB motoneurons, but no contralateral motoneurons (Figure 5.2). Most importantly, in no case did treatment with any gonadal steroid prevent motoneuron depletion. While this is in agreement with my previous studies (Fargo and Sengelaub, 2004a, 2004b), it needed to be confirmed in these studies, and in particular in the ventral root avulsion study, as steroid treatment is known to rescue motoneurons from death in some experimental paradigms (e.g., Ahlbom et al., 2001; Hammond et al., 2001; Huppenbauer et al., 2005; Pike, 2001; Ramsden et al.,



2003). The importance of this finding is that it allows me to state that any potential beneficial effect of steroid treatment on remaining SNB motoneurons cannot be attributed to an attenuation of motoneuron depletion.

Muscle weight data was collected in every experiment. It was included in the experimental designs primarily as a manipulation check, but sometimes revealed interesting relationships. The first observation I would like to point out about muscle weight is that saporin injections had a reliably specific effect. Unilateral saporin injection into the BC and LA muscles reduced BC/LA weight only on the injected side (Figure 6.2). This finding is in concert with my previous studies, in which saporin was also injected unilaterally into the BC and LA (Fargo and Sengelaub, 2004a, 2004b). Moreover, unilateral saporin injection into the BC muscle reduced the weight of the BC but not the (uninjected) ipsilateral LA muscle (Figure 3.1). Given that some researchers consider the LA muscle simply a compartment of the BC, this is a remarkable level of specificity. Similarly, unilateral ventral root avulsion resulted in marked atrophy of the ipsilateral BC/LA, but had no effect at all on contralateral BC/LA muscle weight (Figure 5.1).

In contrast, steroid treatments had highly variable effects on muscle weight. This combination of a reliable depression of muscle weight and a variable effect of steroid treatment led to some interesting observations about the potential relationship between muscle weight and motoneuron morphology in steroid-treated animals. In all of the motoneuron depletion studies that I have conducted so far, testosterone treatment is only effective in protecting the morphology of remaining SNB motoneuron dendrites or somata when it is also effective in increasing the weight of the muscles to which those

motoneurons project. For example, in contralateral saporin injection experiments, delayed treatment with exogenous testosterone both induced hypertrophy in the non-injected muscles and prevented dendritic retraction (Fargo and Sengelaub, 2004a), and immediate treatment with testosterone induced hypertrophy of the non-injected muscles and prevented both somatic and dendritic regression of SNB motoneurons (Fargo and Sengelaub, 2004b; Chapter 6, Figures 6.2, 6.4, and 6.6). In contrast, in the unilateral experiment described in Chapter 3, treatment with testosterone did not produce significant hypertrophy of the non-injected muscle (Figure 3.1), and also failed to prevent saporin-induced dendritic retraction (Figure 3.6). This pattern of results suggests the hypothesis that the ability of steroid treatment to protect dendritic morphology is dependent upon muscle hypertrophy. However, the results reported in Chapter 6, particularly with regard to the DHT-treated group, undermine this idea; DHT treatment actually *decreased* muscle weight slightly (Figure 6.2), while completely preventing saporin-induced dendritic retraction (Figure 6.6). The results reported in Chapter 6 also clearly suggest that androgens, but not estrogens, are capable of protecting SNB dendritic and somatic morphology following contralateral saporin-injection (Figures 6.4 and 6.6).

The results of the ventral root avulsion study (Chapter 5) are more difficult to interpret, primarily because VRA failed to cause somal or dendritic atrophy (Figures 5.3 and 5.6). It is therefore impossible to reach a conclusion, based only on the results of that study, as to whether testosterone's beneficial effects on the morphology of surviving motoneurons generalize to situations in which there is no exogenous neurotoxin introduced into the spinal cord. This study also produced another surprising result: testosterone treatment increased the weight of the denervated musculature above that of

untreated VRA animals (Figure 5.1). This result directly contradicts the two previous reports in the literature that deal with testosterone treatment given to animals with a denervated BC/LA muscle complex (Burešová et al., 1972; Rand and Breedlove, 1992); both of these reports claimed that testosterone treatment had no effect at all on the weight of the denervated musculature. However, there were some differences between those experiments and the current work, which may explain the discrepancy in results; for example, Burešová et al. (1972) only recorded muscle weights for the LA, and Rand and Breedlove (1992) used local rather than systemic testosterone treatment.

The results presented in Chapter 4 demonstrate that saporin injection results in a number of electrophysiological changes, in addition to its effects on the morphology of neighboring motoneurons. Specifically, saporin injection decreased background activity, increased both response latency and activity duration, and depressed SNB motoneuron recruitment (Figures 4.5, 4.6, 4.7, and 4.8). More importantly, the results reported in Chapter 4 suggest that treatment with exogenous testosterone, in addition to maintaining normal motoneuron morphology, protects normal levels of SNB motoneuron recruitment (Figure 4.8). This effect was evident at high response magnitudes, making it all the more relevant for the normal functioning of the SNB neuromuscular system.

#### What does it mean?

A few major conclusions can be drawn from the present work. First, given that testosterone can be metabolized into an estrogen, a major question arising from my earlier work was whether the neuroprotective effect of testosterone was an androgen effect, an estrogen effect, or both. The results reported in Chapter 6 clearly suggest that testosterone's ability to protect SNB dendrites and somata following saporin injection is

an androgen effect. While the non-aromatizable androgen DHT fully protected motoneuron morphology, estradiol had no beneficial effect at all (Figures 6.4 and 6.6). Second, these data indicate that steroid neuroprotection of dendrites and somata is not dependent on muscle hypertrophy, as DHT exerted a beneficial effect on motoneuron morphology without coincident hypertrophy of the target muscles (Figure 6.2). Third, the results reported in Chapter 4 suggest that testosterone treatment exerts a protective effect on the electrophysiological response properties of SNB motoneurons following saporin injection. Specifically, testosterone protects motoneuron recruitment at functionally relevant levels of response magnitude—an effect that appears in concert with, and possibly because of, testosterone’s protective effects on SNB motoneuron morphology (Figure 4.8).

These findings increase the potential usefulness of steroid treatment in neurodegenerative diseases or following traumatic injuries that lead to motoneuron depletion. DHT has much narrower physiological effects than its precursor, testosterone, and so would probably induce fewer off-target effects. As a concrete example from this thesis, DHT can be used in situations in which muscle hypertrophy is not a desirable outcome. In addition, the SNB recruitment data demonstrate that the anatomical benefits of steroid treatment are accompanied by a functional improvement in the electrophysiological response properties of the reflex pathway. Note that these same characteristics, in addition to making steroid treatment attractive as a potential therapy, increase the usefulness of this model for basic research in neuroprotection.

However, the work presented in this thesis also raises new questions as to the effectiveness of using steroids as therapeutic agents following partial motoneuron

depletion. First, the experiment described in Chapter 3 indicates that testosterone treatment is completely ineffective in maintaining the dendritic length of LA-projecting motoneurons following saporin injection into the ipsilateral BC muscle (Figure 3.6). Second, the data presented in Chapter 5 leave me unable to draw a definite conclusion as to whether steroid treatment has a beneficial effect on motoneuron morphology when atrophy is induced by motoneuron loss unrelated to exogenous toxins. While the surprising results of these experiments do not rule out the potential utility of steroid treatment as a therapy, they do beg for more experiments.

### Future Directions

Future studies need to address the concerns raised by the results presented in Chapters 3 and 5. For example, the primary purpose of the experiment reported in Chapter 5 was to determine whether testosterone would be protective in a model of motoneuron depletion that did not involve the introduction of an exogenous neurotoxin into the spinal cord. But the chosen method, ventral root avulsion, did not induce atrophy in the surviving motoneurons. This surprising result left me unable to make a definitive statement regarding the main hypothesis. However, it raises questions as to *why* VRA did not induce atrophy in surviving motoneurons. The experiment leaves open several possible explanations for why saporin injection causes atrophy when VRA does not. For example, the atrophy may result from the direct action of saporin on the remaining motoneurons in the spinal cord, from the necrotic (as opposed to apoptotic) death induced by saporin, or from other possible sequelae of saporin injection, such as the death of primary afferents. By extension, then, testosterone may only have a protective effect in these limited instances. But these conclusions cannot be made on the basis of these data

alone, because there may be other differences between motoneuron depletion by VRA and motoneuron depletion by saporin injection. For example, it could be that the longer, more progressive course of motoneuron death following VRA did not leave sufficient time for contralateral atrophy to manifest. Follow-up experiments could be done in which these variables are manipulated one at a time. Additional information could be gleaned from experiments that precisely determine the time course of VRA-induced motoneuron death in the SNB, then examine contralateral motoneuron morphology 4 weeks after depletion has reached the same levels that saporin causes within the first few days. Of course, it might be wise to consider simply trying another method to kill motoneurons unilaterally without introducing exogenous toxins—such as unilateral contusion. Further, consideration should be given to which method for killing motoneurons, saporin injection or VRA, represents a better model for neuronal depletion in the clinical setting. Various factors should be kept in mind in making such a determination. For example, many neurodegenerative diseases, with ALS, Parkinson's disease, and Alzheimer's diseases amongst them, result in neuronal depletion via apoptosis, which is induced by VRA but not saporin injection. Additionally, many lumbosacral spinal cord injuries are caused by shearing of the spinal roots, a situation much better modeled by VRA than by saporin injection. On the other hand, saporin injection causes motoneuron death by necrosis, which is common following contusion injuries of the spinal cord.

The results from Chapter 3 are similarly puzzling; *why* didn't testosterone treatment prevent atrophy of LA-projecting motoneurons following saporin injection into the ipsilateral BC? This problem is difficult because the experimental setup in this study differed from previous studies in several ways (see pages 48 to 50), any of which could

be responsible for the lack of a neuroprotective effect of testosterone. Initial follow-up experiments should focus on manipulating these variables one at a time. Specifically, I suggest an experiment in which saporin is injected unilaterally into both the BC and LA, then the morphology of contralateral LA-projecting motoneurons is measured in untreated and testosterone-treated animals. This experiment would determine whether the lack of a testosterone effect on dendrites in Chapter 3 was due to the fact that I measured morphology in LA-projecting instead of BC-projecting motoneurons. Depending on the results of that experiment, it might be profitable to perform an experiment in which saporin is injected unilaterally into the LA, and the morphology of ipsilateral BC-projecting motoneurons is measured, again in both untreated and testosterone-treated animals. Assuming unilateral saporin injection into only the LA would be a strong enough manipulation to induce atrophy in BC-projecting motoneurons, this experiment would help determine whether the lack of a testosterone effect on dendrites in Chapter 3 was related to the fact that morphometry was performed on motoneurons ipsilateral to the partial depletion. The results from this chapter reinforce the idea that the LA is not merely the dorsal aspect of the BC muscle. Indeed, LA-projecting SNB motoneurons are probably different from BC-projecting motoneurons in several very interesting ways that have yet to be determined. Delineating these differences could lead to years of exciting research possibilities in this system.

Of course, most motoneurons in the body are different from both BC- and LA-projecting motoneurons in several known ways. First, SNB motoneurons are medially located and innervate a midline muscle complex. While this by no means makes them unique, it is the case that most motoneurons in the body are located in the lateral motor

columns and innervate lateral muscle groups. Second, SNB motoneurons are uniquely sensitive to steroid manipulations. As reviewed in Chapter 1, SNB motoneurons depend on steroids for their survival, development, and adult maintenance. Therefore, a potential criticism of my work is that steroid treatments might not be neuroprotective in motoneuron populations that are not so uniquely steroid-dependent. For these reasons, a critical future direction for this research is to determine whether steroid treatment is similarly protective following motoneuron depletion in other motoneuron populations. In fact, such a project is currently ongoing in our laboratory. In this paradigm, motoneurons are depleted unilaterally by injection of saporin into a thigh muscle; then morphology is assessed in motoneurons projecting to a different thigh muscle, in both untreated and testosterone-treated animals. Preliminary unpublished results indicate that testosterone treatment does indeed preserve normal motoneuron morphology in this experimental design.

Of course, the experiments reported in Chapters 4 and 6, while more easily interpretable, also invite further studies. For example, the results from Chapter 4 suggest that testosterone exerts a protective effect on the function of the SNB by maintaining motoneuron recruitment at high response magnitudes. However, whether this has functional consequences in the awake, behaving animal is still an open question. Given that the primary purpose of the SNB neuromuscular system is to aid in successful copulation (Sachs, 1982), possible follow-up experiments could focus on copulatory behaviors and rates of impregnation. Similarly, the results of Chapter 6 support the hypothesis that testosterone's protective effect is an androgen effect, but they are not completely conclusive. Recent reports suggest that DHT, while non-aromatizable, can be



converted into a metabolite that acts via the beta estrogen receptor (Lund et al., 2004; Pak et al., 2005), raising the possibility, however remote, that testosterone and DHT are exerting estrogen effects after all. Therefore, follow-up experiments should involve using various antiandrogens and antiestrogens to selectively block steroid receptors in testosterone-treated animals.

In conclusion, the results of this dissertation work have shed some light on the use of testosterone as a neuroprotective/neurotherapeutic agent following partial motoneuron depletion, but as is so often the case, they raise more questions than they answer. Going forward, future work should focus on shoring up the arguments presented in this thesis and further delineating the parameters in which steroid treatment is an effective therapeutic strategy.

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