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Review

Neuroprotective actions of androgens on motoneurons

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ABSTRACT

Androgens have a variety of protective and therapeutic effects in both the central and peripheral nervous systems. Here we review these effects as they related specifically to spinal and cranial motoneurons. Early in development, androgens are critical for the formation of important neuromuscular sex differences, decreasing the magnitude of normally occurring cell death in select motoneuron populations. Throughout the lifespan, androgens also protect against motoneuron death caused by axonal injury. Surviving motoneurons also display regressive changes to their neurites as a result of both direct axonal injury and loss of neighboring motoneurons. Androgen treatment enhances the ability of motoneurons to recover from these regressive changes and regenerate both axons and dendrites, restoring normal neuromuscular function. Androgens exert these protective effects by acting through a variety of molecular pathways. Recent work has begun to examine how androgen treatment can interact with other treatment strategies in promoting recovery from motoneuron injury.

1. Introduction

From cellular and molecular perspectives, the consequences of gonadal steroid action in the nervous system include profound trophic effects on target neurons. During development, gonadal steroids are involved in the establishment of sex differences in neural structure and organization that underlie sex differences in behavior [122], promoting the differentiation of a masculine pattern of neural organization [3,111]. For example, androgens have been shown to exert a potent influence on the determination of sex differences in neuron number [116,133,134]. Gonadal steroids also exhibit a wide array of neuroprotective and neurotherapeutic effects on a variety of neuronal populations [4,124]. In this case, the ability of androgens to impact the survival of neurons after injury or in disease, and to promote both neuronal plasticity and regeneration in the context of dendritic or axonal damage, has established androgens as neurotherapeutic agents with clinical potential in the treatment of both peripheral and central neural injuries [148].

The neuroprotective and neurotherapeutic effects of androgens have been the most thoroughly described and documented in motoneuron populations, including cranial motoneurons, sciatic motoneurons, and pudendal motoneurons. In this review, we summarize this work in motoneurons, describing androgenic action in a variety of phenomena, models, and species.

2. Protection from cell death: sexual differentiation

The death of neurons during normal development is a well documented phenomenon that occurs throughout the nervous system. Initially, neurons are overproduced and their numbers are subsequently reduced during a period of normally occurring cell death. The magnitude of death can range up to 80% of the initial neuron population in a given structure, and this death typically occurs over a brief period of time. Normally occurring neuron death contributes to the formation of a variety of structural specializations throughout the nervous system, creating local differences in neuron numbers that underlie functional specializations ranging from high acuity vision to sex differences [132]. In addition, neuron death is thought to establish appropriate ratios of cells in interconnecting populations, as well as to eliminate neurons that have established erroneous connections through diffuse growth of axons.

Abundant evidence exists in the neuroendocrine literature regarding the ability of gonadal steroids to positively impact neuronal viability during development. The first and most thoroughly studied example of the ability of androgens to protect neurons from normally occurring cell death during development comes

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from the sexual differentiation literature. The fifth and sixth lumbar segments of the spinal cord of the rat contain two sexually dimorphic motoneuron nuclei which innervate striated penile muscles. The smaller of these is the spinal nucleus of the bulbocavernosus (SNB), a medially located nucleus containing approximately 200 motoneurons in the adult male. This nucleus is also referred to as the dorsomedial nucleus (DM [131]). In males, SNB motoneurons innervate the bulbocavernosus and levator ani muscles of the penis [10,131], as well as the external anal sphincter [103]. The SNB in mature females, who lack or have greatly reduced perineal musculature [60,19,151], is comprised of only approximately 60 motoneurons, innervating primarily the external anal sphincter [103,153]. Lateral to the SNB lies the sexually dimorphic dorsolateral nucleus (DLN), a much larger nucleus than the SNB, with approximately 600 motoneurons in the adult male but only half as many motoneurons in females [75]. The motoneurons of the DLN innervate the ischiocavernosus muscle of the penis as well as the urethral sphincter [131,75,106]. The ischiocavernosus muscle is also reduced or absent in adult females and thus, as in the SNB, females have a smaller number of DLN motoneurons. These muscles and the motoneurons innervating them control penile reflexes important for copulatory behavior [129,59,63].

This sex difference in SNB and DLN motoneuron number develops perinatally. Before birth, the number of motoneurons in the SNB increases in both male and female rats, the result of a migration of developing SNB cells into their characteristic medial location [133]. SNB motoneuron numbers reach their maxima in both sexes just before birth, and this peak is followed by a decline through postnatal day (P) 10, when cell number reaches its adult range (Fig. 1). The decline is due to sexually dimorphic cell death, as revealed by counts of degenerating cells in the SNB [116]. Females typically lose up to 70% of their SNB motoneurons, whereas males lose about 25%. This sexually dimorphic motoneuron death is dependent on Bax, the pro-death member of the Bcl-2 gene family, and Bax depletion rescues SNB motoneurons [66]. In the DLN, large numbers of motoneurons are present in both males and females embryonically, and decline through P10 [134]. As in the SNB, this decline is due to the death of motoneurons; females typically lose over 70% of their DLN motoneurons, whereas males lose only about 50%.

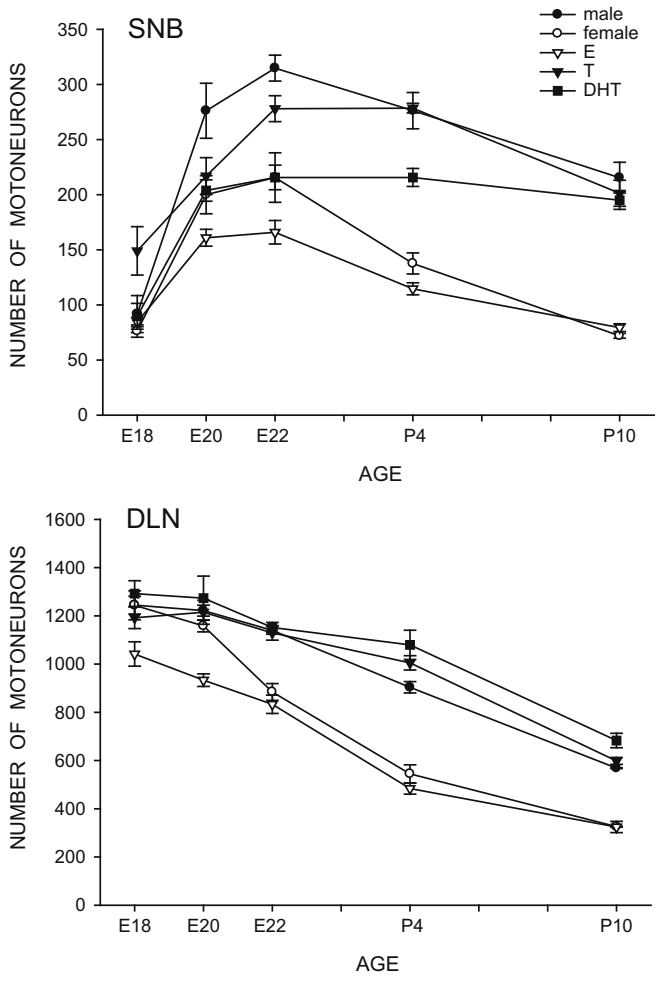

Fig. 1. Counts of motoneurons in the SNB (top) and DLN (bottom) from embryonic (E) day 18 through postnatal (P) day 10 for normal males and females, as well as females treated with estradiol (E), testosterone (T), or dihydrotestosterone (DHT). Points represent means ± SEM. (Compiled from data originally published in [134,136], and Goldstein and Sengelaub, unpublished.)

Gonadal hormones act in the establishment of sex differences in SNB and DLN motoneuron number by regulating this normally occurring motoneuron death. Females treated perinatally with testosterone propionate have reduced cell death during development and significantly more SNB and DLN motoneurons in adulthood than do normal females [13,75,116,133,134]. Conversely, male rats with testicular feminization mutation (*Tfm*, a loss-of-function mutation of the androgen receptor gene) experience a feminine pattern of cell degeneration in the developing SNB [135], and exhibit a feminine number of motoneurons in adulthood [11]. These genetic males (XY) develop functional testes that secrete normal levels of testosterone yet remain unresponsive to the steroid because of greatly reduced androgen binding relative to normal males [113]. Prenatal treatment with the anti-androgen flutamide, a drug known to block androgen receptor activation, also feminizes SNB motoneuron number in males [12].

This effect of androgens in the regulation of motoneuron survival during early development has also been observed in the sexually dimorphic laryngeal motor nucleus in African clawed frogs [79]. These cranial motoneurons are part of a sexually dimorphic neuromuscular system involved in vocalization, and the greater number of motoneurons in males is thought to be due to an androgen-regulated developmental cell death. While treatment of male tadpoles with androgens has no effect, it increases the number of laryngeal motoneurons in females [79].

Androgens are thought to regulate the death of SNB motoneurons indirectly through their action at the target musculature. While treatment with androgens in the late prenatal or early postnatal period reduces SNB motoneuron death, SNB motoneurons do not express androgen receptors until the second postnatal week [44,76,77]. In addition, the normal androgen-induced masculinization of the SNB can be prevented by local application of the anti-androgen flutamide to the target muscles [43]. Studies implicating the SNB target muscles as the site of action have demonstrated that these muscles can be maintained by androgen treatment during development even if they have been denervated by removal of the lumbar spinal cord at birth [42], and moreover, the muscles possess androgen receptors at birth [44]. In a study by Freeman et al., [50], *Tfm* females who were genetically mosaic for androgen insensitivity were treated with testosterone during the period of SNB motoneuron death. These researchers capitalized on the fact that the androgen receptor gene resides on the X-chromosome [160]. As a result of the random X-chromosome inactivation that occurs in every cell of XX embryos during development, female carriers of the *Tfm* mutation have some SNB cells that express the wild-type androgen receptors, and some that express the *Tfm* androgen receptors. Despite lacking functional androgen receptors, SNB motoneurons expressing the *Tfm* allele were spared by early testosterone treatment [50]. Because the SNB target musculature was also spared in these females, and these muscles contained

functional androgen receptors, it seems plausible that androgen action in the neuromuscular periphery is responsible for the indirect support of SNB motoneuron survival.

Many of the actions of testosterone occur through its conversion to the non-aromatizable androgen dihydrotestosterone (DHT) or estrogenic metabolites [65]. For example, in rats, the sexually dimorphic nucleus (SDN) of the preoptic area of the hypothalamus is larger in adult males than in females. This sex difference is the result of estrogenic action during development. Females treated with estradiol perinatally develop a masculine SDN [27], while treatment of males with estrogen antagonists results in a feminine SDN [29]. Furthermore, DHT treatment is ineffective in masculinizing the SDN in female rats, and males given androgen antagonists perinatally develop an SDN that is no different from those of normal males [28]. Similarly, DHT treatment of female mice fails to completely masculinize the number of calbindin-immunoreactive neurons in the SDN [6].

Unlike SDN neurons, the protection from normally occurring motoneuron death during development appears to be strictly androgenic. Animals treated with DHT throughout the pre- and postnatal critical period have an SNB system that is completely masculine in terms of motoneuron number (Fig. 1) and presence of the target muscles [56]. Prenatal treatment with DHT is sufficient to completely masculinize DLN motoneuron number [136]. By contrast, in both the SNB and DLN, treatment of females with estradiol benzoate completely fails to alter cell number from the normal feminine pattern [15,55,9] (Fig. 1). Similarly, SNB motoneuron number is not affected in males treated postnatally with the aromatase inhibitor 4-OH-androstenedione [24], indicating that conversion of testosterone to an estrogen is not necessary for the development of a masculine number of motoneurons in this system. The findings in *Tfm* rats, and the failure of estrogens to prevent SNB motoneuron death, suggests that the sparing of both the SNB and its target musculature specifically requires activation of the androgen receptor.

Androgenic hormones may be required to stimulate the production of trophic factors from the neuromuscular periphery that then mediate the rescue of SNB motoneurons. Although these factors have not been identified, injection of ciliary neurotrophic factor into the perineum of newborn females spares SNB motoneurons [47], and the ability of testosterone to masculinize SNB motoneuron number is blocked by perineal injections of antagonists to trophic factors [156].

3. Protection from cell death: injury

Gonadal steroids have been demonstrated to exert neuroprotective effects after nervous system trauma or disease, and there is substantial data in the literature that gonadal steroids can rescue neurons from cell death following a wide variety of neurological insults [155,4,124]. The molecular bases for gonadal steroid-mediated effects on neuron viability are quite intriguing; these effects can involve both androgenic and estrogenic mechanisms, and can be mediated by classical steroid receptors or by mechanisms independent of intracellular receptor populations.

In the 1990s, converging evidence from animal experiments and epidemiological studies indicated that estrogens can act as neuroprotectants in the central nervous system [52]. Less was known about the ability of androgens to act as neuroprotectants, however, until late in the decade, when the results of *in vitro* experiments demonstrated that cerebellar granule neurons can be protected from oxidative stress by androgens [1,2]. Testosterone was also shown to protect primary cultures of human neurons from cell death caused by serum deprivation [58] and cultured rat hippocampal neurons from beta-amyloid toxicity [123]. Androgens also protect cultured immortalized motoneurons from heat shock-induced cell death. Since these neuroprotective effects of androgens on cultured motoneurons occur in the absence of glial elements or synaptic input, the molecular mechanisms underlying rescue appear to be neuronally based [148].

Subsequent *in vivo* work has demonstrated that androgens are also capable of preventing neuronal cell death in the brain after various types of injury. For example, facial nerve transection kills facial motoneurons in neonatal hamsters, but androgen treatment prevents this [64]; and androgens can enhance neuronal viability after kainate lesions in rat hippocampus [126]. Similarly, treatment with DHT reduces laryngeal motoneuron loss after axotomy [120].

4. Axonal injury

In mammals, axons in the injured central nervous system generally do not regenerate well. In contrast, axons in the peripheral nervous system are known for their remarkable regenerative capacity. As long as the connective tissues of the nerve sheath are left relatively intact, most peripheral axons readily regrow and reinnervate their original targets with a high degree of specificity. This is true of both cranial and spinal motoneurons. However, the regenerative capacity of peripheral motoneuron axons is not limitless. Both severity of the nerve injury and proximity of the injury to the cell body can lead to relatively poor nerve regeneration [142]. It is therefore important to uncover the factors that determine the regenerative ability of injured neurons, and begin to understand how to enhance these abilities. In fact, a significant amount of research has been done on these topics [95,109], and androgens have proven to be effective agents in enhancing nerve regeneration.

5. Androgen-enhanced nerve regeneration

In the 1980s, several laboratories described the positive actions of androgens on neuronal regeneration in different motoneuron injury paradigms. Yu and collaborators used the rat hypoglossal motoneuron as one experimental paradigm [162,163], while others utilized spinal nerve injury models [154,161]. Early studies by Yu and colleagues demonstrated a sex difference in the rates of axon regeneration (males regenerating faster) following axotomy of the hypoglossal nerve in rats [162], and that androgen treatment enhances axon regeneration rates in both male [163,167] and female [166] rats.

One of the most useful *in vivo* models of axon regeneration is the rodent facial nerve axotomy model [68,109]. Injury to the facial nerve at its exit from the skull through the stylomastoid foramen leaves the blood–brain barrier intact and does not directly damage the cell bodies within the brainstem. Facial nerve injury results in a characteristic unilateral facial paralysis, associated with drooping of one corner of the mouth, flattened and paralyzed vibrissae, and loss of the eyeblink reflex. In rodents, complete recovery of gross motor movements is nearly always observed within 3 weeks.

Rodent facial motoneurons contain androgen receptor mRNA and protein [164,30] and, therefore, represent an ideal system in which to study the effects of androgens on peripheral nerve repair. The first indication that androgens might regulate axon regeneration in the facial nerve was in a series of experiments published in 1989 [85]. In this paper, the authors reported experiments in which adult male Syrian hamsters were subjected to unilateral facial nerve crush, then treated with various regimens of testosterone propionate and evaluated for the length of time until recovery of facial motor function. The results demonstrated that testosterone exerts a dramatic effect on the speed of functional

recovery. Furthermore, the experiments revealed a dose–response relationship, with higher doses and more frequent treatments producing better results; the steady dose achieved by Silastic implants yielded the best results. Androgen treatment also accelerates functional recovery from facial nerve crush in mice [148].

The initial finding that testosterone accelerates functional recovery following facial nerve injury led to the hypothesis that testosterone might exert its beneficial effect by increasing the temporal rate of regeneration of the injured axons. To test this hypothesis, Kujawa and colleagues [82] injected tritiated amino acids into the facial motor nuclei of male and female hamsters with facial nerve crush injuries. Because these radiolabeled amino acids were incorporated into protein and transported to the growing tips of the axons by fast axonal transport, it was then possible to measure the distance traveled by the fastest growing axons within the facial nerve. Testosterone administration led to a 26–30% increase in the rate of regeneration in males (Fig. 2). Interestingly, baseline axon regeneration rates were higher in females than in males. Testosterone also increased axon regeneration rates in females, but to a lesser degree than in males. There is a corresponding sex difference in speed of functional recovery following facial nerve crush, with females recovering before males [67]. Testosterone treatment speeds functional recovery in males, but has no significant effect on speed of recovery in females [67].

This finding of a sex difference in testosterone-enhanced axon regeneration suggests that the facial nerve systems of male and female hamsters may be differentially responsive to androgens, perhaps due in part to an inherent sex difference in basal androgen receptor levels. This hypothesis is strengthened by the fact that administration of flutamide, a potent androgen receptor blocker, completely abolishes the ability of testosterone to enhance facial nerve regeneration rates, indicating that the effect of testosterone is indeed androgen receptor-dependent [86]. Additionally, DHT, which is often found to be a more potent ligand for androgen receptors than testosterone, produces a greater acceleration in regeneration than testosterone [145]. Drengler and colleagues [30] provided evidence for a sex difference in androgen receptor levels by demonstrating that female hamsters express androgen receptor mRNA in their facial motoneuron somata at only about half the levels that male hamsters do. It is unknown whether this difference in message is translated into a difference in protein, but if so, this could account for the reduced effect of testosterone on axon regeneration rates in females. There is a known sex difference in androgen receptor protein in rat facial motoneuron somata, with males having more [165]. Interestingly, while testosterone treatment of castrated males increases androgen receptor mRNA in facial motoneuron somata, this effect is completely blocked by axotomy [31,89]. Axotomy-induced downregulation of androgen receptor has also been found in spinal motoneurons [92,157]. These findings suggest that the regulation of androgen receptor levels in motoneuron somata depends at least in part on a peripherally-derived factor. More importantly, however, they suggest that the accelerative effects of testosterone on axon regeneration do not depend on increased transcription of androgen receptor message in the regenerating motoneuron.

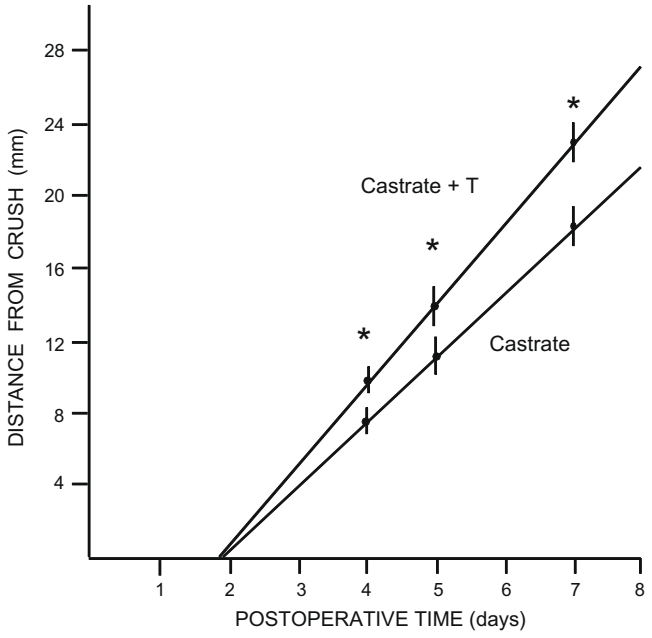

Fig. 2. Outgrowth distances from the point of nerve crush to the leading edge of the growing axons following facial nerve crush in castrated male hamsters, either treated with testosterone (T) or left untreated. Circle heights and bars represent means ± SEM. * indicates $p < .05$. Diagonal lines represent extrapolation of the data (data from [82]).

Androgen enhancement of facial nerve regeneration has important temporal requirements. An early study of the effects of testosterone on the recovery of facial motor function following crush injury suggests that the first week following injury represents a "critical period" [84]. If testosterone treatment is delayed until 6 days after the injury, it is ineffective in accelerating recovery. However, if testosterone treatment is given immediately, it is beneficial even if withdrawn after 7 days, well before full behavioral recovery. In fact, the effective temporal window of testosterone is actually much shorter than this [146]. Administration of testosterone for only 6 h post-injury has the same effectiveness on both functional recovery and axon regeneration rates as does continuous administration of testosterone throughout the full recovery phase. Moreover, delaying administration of testosterone until 6 h after injury completely eliminates its effectiveness, even if it is administered continuously from that point forward.

Testosterone has also been shown to accelerate functional recovery from hind limb paralysis following sciatic nerve crush in rats [16], and to enhance sciatic axon regeneration rates [83]. In view of the importance of motoneuron viability to a successful outcome from spinal cord injury, this is a matter of critical therapeutic significance. Castrated male rats were subjected to a mid-thigh crush injury of the sciatic nerve (approximately 65 mm from the spinal cord), then treated with either testosterone or vehicle. Systemic exposure to androgens accelerated axon regeneration rates, but did not alter the delay before sprout formation occurred. This acceleration has important consequences for recovery, as rats given sustained-release testosterone implants recover from axotomy-induced hind limb paralysis more quickly than untreated rats [15]. The superior efficacy of implants over endogenous hormone in supporting androgen-dependent features has been observed previously (e.g., [25]), and may reflect the difference between a given (and fixed) circulating testosterone level from an implant and the same average (but fluctuating) level of hormone in intact males.

Interestingly, the sciatic nerve has been shown to express androgen receptor [94], apparently in the endoneurium [78], raising the possibility that androgens exert some of their beneficial effects on nerve regeneration by acting in the periphery.

Interestingly, androgen enhancement of functional recovery following peripheral axotomy appears to depend in part on route of administration. When testosterone is administered by subcutaneous injections rather than sustained-release implants, the steroid can still enhance axon regeneration in the sciatic nerve, but has no beneficial effects on functional recovery [144]. Similarly, sustained-release testosterone decreases functional recovery

times following facial nerve axotomy, but injected testosterone does not [85].

6. Axotomy effects on dendrites

After peripheral axotomy motoneurons show a range of responses including structural, functional, and biochemical changes (e.g., [57,150,5]. For example, axotomy of sciatic motoneurons by nerve crush causes dendritic retraction after 2 months [117]. Axotomy also changes the electrophysiological properties of motoneuron dendrites, for example, giving rise to novel sodium-dependent partial spikes [137]. Permanent axotomy of gastrocnemius motoneurons reduces dendritic diameter within 3 weeks, and dramatically reduces dendritic membrane area and volume within 12 weeks [7]. Actual disconnection of motoneurons from their target musculature is not required to induce dendritic retraction; for example, chemical blockade of functional contact between hypoglossal motoneurons and the tongue results in dendritic retraction [141]. The dramatic regressions that occur in motoneuron dendritic arbors after axotomy can be reversed upon muscle reinnervation [141,6,8,117]. This association between dendritic arbor size and muscle contact suggests that target musculature provides some sort of trophic support for motoneurons.

7. Protective effects on dendrites after axotomy

In addition to its effects on nerve regeneration, testosterone has been shown to have a variety of protective effects on other aspects of motoneuron structure and function. Treatment with either testosterone or brain derived neurotrophic factor (BDNF) alone can prevent axotomy- or castration-induced declines in soma size of SNB motoneurons [158]. Furthermore, treatment with testosterone and BDNF shows an interactive effect in the regulation of androgen receptor expression. Axotomy of adult SNB motoneurons causes a dramatic decline in the expression of androgen receptor immunoreactivity [157]. While application of BDNF alone to the cut SNB axons is ineffective in preventing this decline, combined treatment with both testosterone and BDNF is more effective than treatment with testosterone alone [157]. Androgen and BDNF show a similar interactive effect in the maintenance of dendritic morphology [159]. SNB dendritic lengths are dramatically reduced in axotomized castrated males, and treatment with either testosterone implants or BDNF alone applied to the cut axons is ineffective in preventing similar declines in dendritic length (Fig. 3). However, combined treatment with both testosterone and BDNF fully supports SNB dendritic morphology after axotomy. The requirement for testosterone may be at least partially due to the fact that the expression of the BDNF receptor, trkB, in spinal motoneurons is regulated by testosterone: motoneurons of castrated males deprived of testosterone show reduced expression of trkB receptors compared to motoneurons of intact animals or castrated males given testosterone replacement [118,119].

8. Androgen maintenance of motoneuron morphology

Manipulating androgens in adulthood can have marked effects on motoneuron soma size. Castration of adult males leads to the shrinkage of SNB [11], and to a lesser degree DLN [87], motoneuron somata, which can be prevented by treating castrated males with testosterone. This effect is not universal in motoneurons and likely reflects differences in androgen sensitivity across motoneuron populations [91]. For example, castration with or without hormone replacement has no effect on soma size in quadriceps motoneurons [118].

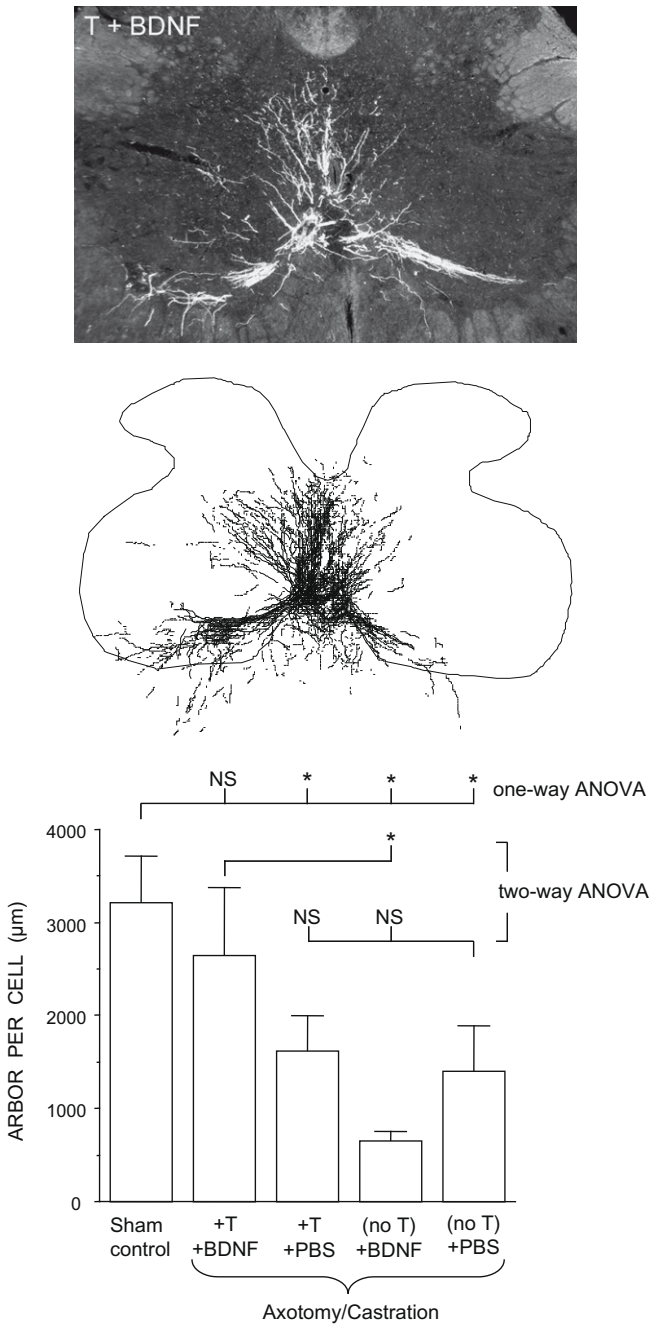

Fig. 3. Darkfield photomicrograph (top) and a computer-generated composite (middle) of BHRP-labeled SNB motoneurons in an axotomized castrated male treated with both testosterone and BDNF. (Bottom) SNB dendritic lengths were dramatically reduced in axotomized castrated males compared to those of intact males. Treatment with either testosterone (T) implants or BDNF applied to the cut axons alone was ineffective in preventing similar declines in dendritic length. However, axotomized castrate males given both testosterone and BDNF had SNB dendritic lengths that did not differ from those of intact males. Bar heights represent means ± SEM. * indicates $p < .05$ (data from [159]).

DHT partially protects SNB cells from shrinkage after castration of adult male rats, but is not as effective as testosterone when given in the same dose [46]. Estrogenic metabolites of testosterone do not seem to explain the difference in efficacy between testosterone and DHT, however, as estradiol alone or in conjunction with DHT has no effect on soma size [46]. Similarly, blockade of estradiol synthesis in intact adult male rats with the aromatase inhibitor, fadrozole, does not affect SNB soma size [18].

A similar pattern of results is seen in gerbils, where long-term castration of adult males leads to a reduction in SNB soma size that can be prevented by treatment with testosterone, but not estradiol [49]. As in rats, DHT is partially effective in preventing the castration-induced reduction in SNB cell size in gerbils [49]. This suggests that testosterone increases SNB soma size of adult rodents by acting via androgen receptors, and may do so without conversion to either estradiol or DHT.

In adulthood, castration results in a substantial reduction in the length of dendrites in both SNB and DLN motoneurons which can be completely reversed with androgen replacement [88,87]. This kind of androgen-dependent, reversible plasticity had not been reported previously in an adult mammalian system, and was particularly exciting because changes in androgen levels occur normally and correlate with changes in the frequency of copulatory behaviors. For example, a naturally occurring analog of hormone depletion and replacement can be seen in seasonally breeding mammals such as white-footed mice. As in rats, castration results in decreased dendritic arbors in these mice [45]. Similarly, circulating testosterone levels decline with advanced aging, and these declines are accompanied by reductions in sexual behavior. Fargo et al., [35] found that the bulbocavernosus and levator ani muscles and their innervating SNB motoneurons underwent profound atrophy with advancing age, with muscle weight and motoneuron dendritic length declining to less than 50% of young adult levels. Treatment of aged animals with testosterone completely reverses the age-related declines in muscle weight and SNB motoneuron morphology, demonstrating that the SNB system retains its androgen-mediated plasticity throughout life.

Concomitant with changes in dendritic length, androgens control the number and size of gap junctions and synapses on SNB motoneurons [90,101,102]. Although the molecular bases for androgenic effects on dendrites, gap junctions or synapses are not fully understood, androgen manipulation directly or indirectly affects the expression of a number of candidate genes and proteins in SNB motoneurons, including those for gap junction proteins [99], N-cadherin [107], calcitonin gene-related peptide [125], androgen receptor [98], the ciliary neurotrophic factor receptor α [48], and the major cytoskeletal elements, β-actin and β-tubulin [99,97].

Rand and Breedlove [127] showed that testosterone can regulate SNB dendrites by acting at the target musculature. In castrated males, SNB motoneurons projecting to testosterone-implanted bulbocavernosus muscles have significantly longer dendritic lengths than those projecting to muscles on the contralateral side treated with the anti-androgen flutamide. This result suggests that, similar to the effects described earlier for hormonal support of dendritic morphology after axotomy [159], androgens regulate a neurotrophic signal from the muscle that is critical in the maintenance of dendritic organization in adulthood.

9. Induced atrophy after motoneuron depletion

Neurodegenerative disease or nerve injury often results in the loss of spinal motoneurons. For example, amyotrophic lateral sclerosis (ALS), the most common motoneuron disease in adult humans, is characterized by the selective death of upper or lower motoneurons (as well as small interneurons) in the brain and spinal cord. This cellular degeneration results in a progressive muscle weakness, atrophy and spasticity, and ultimately a nearly complete paralysis [20]. While ALS is the most prevalent, other diseases including the motor neuron diseases and spinal muscular atrophies (e.g., spinal and bulbar muscular atrophy [80]) are also characterized by progressive loss of motoneurons. Similarly, damage to spinal nerves resulting in laceration and avulsion of spinal roots (e.g., cauda equina injury with high impact motor vehicle accidents [112]) can lead to the death of motoneurons and preganglionic autonomic neurons in the spinal cord, resulting in autonomic and motor dysfunction [62].

However, the death of motoneurons is not the only outcome, and importantly, remaining motoneurons after such insults show a variety of morphological and functional changes. Experimentally-induced partial depletion of motoneurons, produced by injections of the retrogradely transported neurotoxin saporin into the target musculature, results in substantial somal and dendritic atrophy in surviving SNB or quadriceps motoneurons [36,37,39,91]. In both cases, after the death of motoneurons by saporin injection, the cross-sectional area of surviving nearby SNB or quadriceps motoneurons is reduced [36,37,39,91]. Similarly, induced motoneuron death results in a pronounced dendritic atrophy in surviving nearby motoneurons (Fig. 4). Four weeks after partial motoneuron depletion, dendritic length in both SNB and quadriceps motoneurons is decreased by over 60% [36,37,39,91]. Evi-

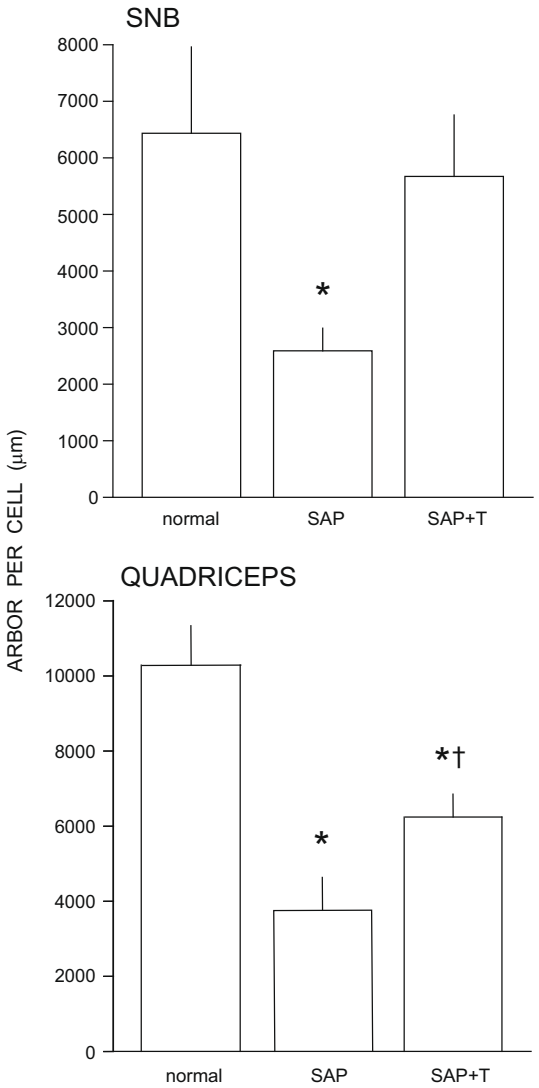

Fig. 4. Testosterone treatment is neuroprotective in SNB (top) and quadriceps (bottom) motoneurons, attenuating induced dendritic atrophy resulting from the death of nearby motoneurons. Motoneuron dendritic lengths are expressed as length of arbor per labeled motoneuron for normal males and saporin-treated males with (SAP+T) or without (SAP) supplemental testosterone. Bar heights represent means ± SEM. * indicates significantly different from normal males. † indicates significantly different from untreated saporin-injected animals (data from [37,91]).

dence of recovery in dendritic lengths is present at 6 weeks post depletion, and by 10 weeks, dendritic lengths recover to those of normal, intact males [40].

These regressive changes in motoneuron morphology are correlated with changes in motor activation: concomitant with atrophy, stimulation-evoked activation of motoneurons is attenuated. In saporin-injected animals, stimulation of the dorsal root afferents to the SNB or quadriceps motoneurons produces responses in the respective pudendal or femoral nerves whose amplitudes are dramatically reduced compared to those of normal males [38,91].

Treatment with testosterone is neuroprotective for remaining motoneurons following partial depletion. In the SNB, testosterone treatment attenuates dendritic atrophy, resulting in dendritic lengths that are reduced an average of only approximately 16% from normal lengths, and are greater than those of saporin-injected animals by an average of over 119% [36,37,39] (Fig. 4). While testosterone treatment is effective in attenuating dendritic atrophy in quadriceps motoneurons, it does not protect them to the same degree as observed in the highly steroid-sensitive SNB [91]. Quadriceps motoneurons in testosterone-treated saporin animals have dendritic lengths that are reduced almost 40% from normal lengths, and are greater than those of saporin-injected animals by only approximately 65% (Fig. 4). Changes in motor activation across the SNB and quadriceps motor populations reflect these differences in the magnitude of the neuroprotective effects of testosterone on dendritic morphology. Stimulation-evoked activation of SNB motoneurons is restored to intact levels with testosterone treatment, but in quadriceps motoneurons testosterone treatment is again only partially protective, resulting in intermediate levels of motor activation [38,91]. A likely explanation for this difference lies in the expression of androgen receptors in these two systems. SNB motoneurons abundantly express androgen receptors, and almost 70% of SNB motoneurons are labeled with tritiated testosterone at 5× background grain density; in contrast, less than 3% reach this criterion in other motoneurons [9,14]. Similarly, the principal SNB target muscles in males (the bulbocavernosus and levator ani muscles) are enriched for androgen binding sites and androgen receptor protein compared to other striated muscles [32,152,108]. Androgen receptor protein is present in substantially higher concentrations in the levator ani compared to other skeletal muscle [108], and the bulbocavernosus and levator ani muscles have over four times as many binding sites for testosterone than are present in the quadriceps muscles [32]. Thus, as differences in the density of androgen receptors are thought to underlie differences in androgen responsiveness across tissues [108], the lower density of androgen receptors in the quadriceps system might result in a smaller protective effect of testosterone. Regardless of the differences in degree, testosterone treatment is neurotherapeutic in both SNB and quadriceps motoneurons, protecting against the reductions in excitability seen after partial motoneuron depletion and providing a functional measure of recovery.

The time course of induced atrophy and recovery from partial motoneuron depletion with saporin is protracted, and treatment with testosterone appears to attenuate the atrophy rather than prevent it. While initial dendritic atrophy (2 weeks after motoneuron depletion) is similar to that of untreated saporin-injected males, testosterone treatment prevents further decrements in length [40]. Furthermore, in both SNB and quadriceps motoneurons this attenuation of dendritic atrophy by testosterone is dose-dependent, and 4 weeks after motoneuron depletion, dendritic lengths in males treated with physiological levels of testosterone are longer than those of untreated, saporin-injected males [23]. Treatment with higher dosages of testosterone does not produce any further attenuation in dendritic length [23].

As described earlier for the effects of androgen on facial nerve regeneration, protection from dendritic atrophy after partial motoneuron depletion with saporin also has important temporal requirements [22]. Four weeks of testosterone treatment (delivered immediately post-saporin), or 2 weeks of testosterone treatment (after a delay of 2 weeks post-saporin) are both effective in attenuating induced dendritic atrophy in quadriceps motoneurons. However, dendritic atrophy in animals with immediate testosterone treatment of shorter durations or longer delays in the start of treatment, is comparable to that of animals who received no supplemental testosterone. This result demonstrates that similar to facial motoneurons, the neuroprotective effect of testosterone on quadriceps motoneuron morphology may also have a "critical period". While motoneuron depletion occurs within a few days after saporin injection, loss of dendrites in remaining quadriceps motoneurons occurs most dramatically between 2 and 4 weeks post-saporin (Coons and Sengelaub, unpublished), the period in which testosterone treatment is maximally effective.

Consistent with most other morphological effects in adult SNB motoneurons, the neuroprotective effects of steroid treatment appear to be strictly androgenic, as treatment with either testosterone or DHT, but not estradiol, attenuates induced somal and dendritic atrophy following saporin injections [39]. By contrast, dendritic atrophy following saporin injections in motoneurons innervating the quadriceps muscles is attenuated to a similar degree by treatment with testosterone or its metabolites (Muñoz et al., unpublished).

10. Cellular and molecular mediators

The mechanisms by which androgens act to impart neuroprotection on selective neuronal populations are currently under investigation by numerous laboratories. In view of substantial data demonstrating neuroprotective effects of estrogens [143] and the widespread distribution of aromatase in the brain [128], one likely molecular scenario includes the conversion of circulating androgens to estrogens *via* brain aromatase [53]. However, there is also recent evidence for direct androgenic activation of neuroprotective pathways *via* both classical androgen receptor and membrane-associated receptors [114,115,54]. These studies suggest that in some cases androgens are able to exert neuroprotective actions without conversion to estrogens.

Understanding the molecular mechanisms by which androgens act on injured neurons is likely to provide important insights for developing strategies for therapeutic interventions for nervous system trauma and neurodegenerative diseases. Several proteins thought to be involved in neuroprotection are regulated by androgens. For example, proteins with antioxidant functions (e.g., catalase [2]) or involved in the regulation of cytoplasmic calcium (e.g., calbindin [121] are androgen-mediated. As discussed above, the neurotrophin BDNF [119] and its receptor trkB [118] are both regulated by androgens. Androgens are also thought to be involved with the activation of signaling pathways involved in neuroprotection (e.g., MAPK/ERK [124]).

Direct axonal damage results in a retrograde cell body reaction as well as significant changes in surrounding glial cells. Androgen receptors are expressed in both neurons and glia [41,51,130], so androgen treatment has the potential for both direct and indirect modulation of the central neuronal repair process. In the case of the facial motor nucleus, axotomy is known to result in a process known as synaptic stripping, in which the synaptic input to the injured motoneurons is dramatically reduced, and neuron–neuron contacts are replaced by glia–neuron contacts [71]. This is accompanied by an upregulation in the facial motor nucleus of the glial marker GFAP at both the mRNA [72] and protein [21] levels. However, androgen treatment attenuates the process of synaptic

stripping, preserving central input to the motoneurons [71], as well as attenuating the axotomy-induced upregulation of GFAP [21,67,69]. Similar effects have also been observed in the rat [109].

Other cellular and molecular elements that might be involved in the neuroprotective effects of androgens include ribosomal RNA, neuronal and glial cytoskeletal proteins, and stress proteins. Intracellular protein synthesis events are mediated by the nucleolar response to injury, and androgen exposure enhances this response. Following facial nerve axotomy in the hamster, testosterone treatment causes ribosomal RNA levels to increase more rapidly and to a greater magnitude than injury alone [81].

GAP-43, a cytoskeletal protein associated with the membranes of axons, is upregulated by axotomy in the peripheral nervous system [147]. Injury of the facial motor nerve increases GAP-43 mRNA and protein in the facial nucleus, and androgen treatment augments this response [21,70]. Other cytoskeletal proteins are also involved in androgen-enhanced nerve regeneration. The primary cytoskeletal structures in axons are microtubules, which are composed of tubulin proteins. Tubulin mRNAs are upregulated in facial motoneurons as the axons regenerate following axotomy [73,74,147]; androgen treatment, which enhances axon regeneration rates, causes an additional upregulation of tubulin mRNAs above axotomy alone [73,74]. Importantly, tubulin mRNAs are upregulated by androgen treatment selectively. While α_I-tubulin, β_{II}-tubulin, and β_{III}-tubulin are all upregulated by axotomy, only β_{II}-tubulin has been shown to be under the control of androgen in this model [73,74]. Tubulin genes are also regulated by androgen in axotomized sciatic motoneurons [17] and rubrospinal motoneurons [140,26], and in the SNB [97,100], raising the possibility that this may be a general phenomenon in motoneurons.

Androgenic enhancement of axon regeneration also appears to be under the control of neuritin. Neuritin mRNA levels are upregulated by androgen treatment in cultured motoneuron-neuroblastoma hybrid cells in an androgen receptor-dependent manner [34], and neuritin has been shown to be a critical downstream mediator of the ability of androgens to increase neurite outgrowth in cultured NSC34/mAR cells [96]. In the injured hamster facial motor nucleus, testosterone causes a ~300% increase in neuritin mRNA levels 2 days post-injury [34]. Because testosterone treatment increases the rate of axon regeneration in injured hamster facial motoneurons [82], these data demonstrate a relationship between neuritin expression and androgen-enhanced axon regeneration *in vivo*. This relationship is further supported by the fact that blocking androgen receptors with flutamide, which is known to block the effects of testosterone on axon regeneration rates [86], completely abolishes the effect of testosterone on neuritin mRNA expression in the injured hamster facial nucleus [34]. The time course of androgenic regulation of neuritin expression in the hamster facial motor nucleus, with the most pronounced effect of testosterone occurring at 2 days post-injury, is particularly interesting for two reasons. First, extrapolation of hamster facial motoneuron axon regeneration data indicates that axon sprouting occurs at about 2 days post-injury [82]. Second, androgen treatment enhances axotomy-induced upregulation of β_{II}-tubulin expression between 2 days and 7 days post-injury [73,74].

Facial nerve injury also results in a heat shock response, in the form of upregulation of HSP70, a protein known to be upregulated in response to a variety of cell stresses, including central nervous system injury [33]. It has been suggested that testosterone treatment exerts its neuroprotective effects in axotomized hamster facial motoneurons in part by modulating this response [148,149]. Unbound androgen receptors in the cytoplasm form protein complexes with various heat shock proteins, and upon androgen binding, these heat shock proteins are released. During the normal cell stress response, there is a temporary cessation of rRNA processing and of overall protein synthesis [110]. Heat shock proteins are usually induced in the cell during this time, in order to complex with intracellular proteins denatured by the stressor. However, exogenous androgens delay the stress response following hamster facial nerve axotomy, presumably because androgen receptor binding releases a pool of immediately available heat shock proteins [149]. This obviates the need to divert the cellular protein synthesis machinery from a regenerative response to a cell stress response. Thus, androgen treatment at the time of injury may allow an uninterrupted axonal repair process to proceed, with the result being marked increases in the rates of axon regeneration and overall functional recovery.

Finally, a significant body of work has emerged pointing to peripheral nerves as targets for steroid actions [93,78,104,105]. These studies suggest that neuroactive steroids can actually be synthesized in nerves, and can work in the nerves *via* both classical and nonclassical steroid receptors. Furthermore, these studies indicate that one molecular target of neuroactive steroids is peripheral myelin, specifically the myelin-related proteins Po and PMP22.

11. Androgens and electrical stimulation

Recently work has begun to explore the effectiveness of combinatorial treatment involving both androgen and electrical stimulation in promoting recovery from peripheral nerve injury in rats. In a typical experiment, rats are subjected to unilateral facial nerve crush injury, treated with testosterone or no steroid, and given electrical stimulation or sham stimulation. Stimulating electrodes are implanted just proximal to the site of crush injury, and short bursts of stimulus pulses are delivered at 1–4 mV while the animals are awake and unrestrained [61]. Using radiolabeled amino acids injected into the facial nucleus, it has been shown that testosterone treatment increases the rate of axon regeneration without decreasing the delay before sprout formation [138]; this result is identical to what has been found in hamsters [82]. In contrast, electrical stimulation decreases the delay before sprout formation, without increasing the rate of axon regeneration. Combinatorial treatment with both testosterone and electrical stimulation both decreases the delay to sprout formation and increases the rate of axon regeneration, resulting in a better outcome than either treatment alone [138]. This result is mirrored in measures of functional recovery from facial nerve injury, where combinatorial treatment with testosterone and electrical stimulation results in faster functional recovery than either treatment alone [61].

Electrical stimulation appears to have a very early effect on axon regeneration, reducing the delay to sprout formation from 2 days to 1 day post-injury, while testosterone has a more sustained effect, increasing the rate of axon regeneration through at least 7 days post-injury [138]. This observation suggests that androgen treatment and electrical stimulation may act on different cellular and molecular processes in enhancing axon regeneration after injury. Expression of several regeneration-associated genes reveals that some of these genes are regulated only by electrical stimulation, some only by testosterone, and some by both [139] (Fig. 5). For example, GAP-43 mRNA levels are increased beyond nerve injury alone by electrical stimulation, but not by testosterone treatment. In contrast, β_{II}-tubulin mRNA levels are increased by testosterone treatment, but not by electrical stimulation. Finally, some genes, such as neuritin, are regulated by both electrical stimulation and testosterone treatment.

12. Conclusions

In summary, it is clear that androgens have robust neuroprotective effects on motoneurons. From the early developmental

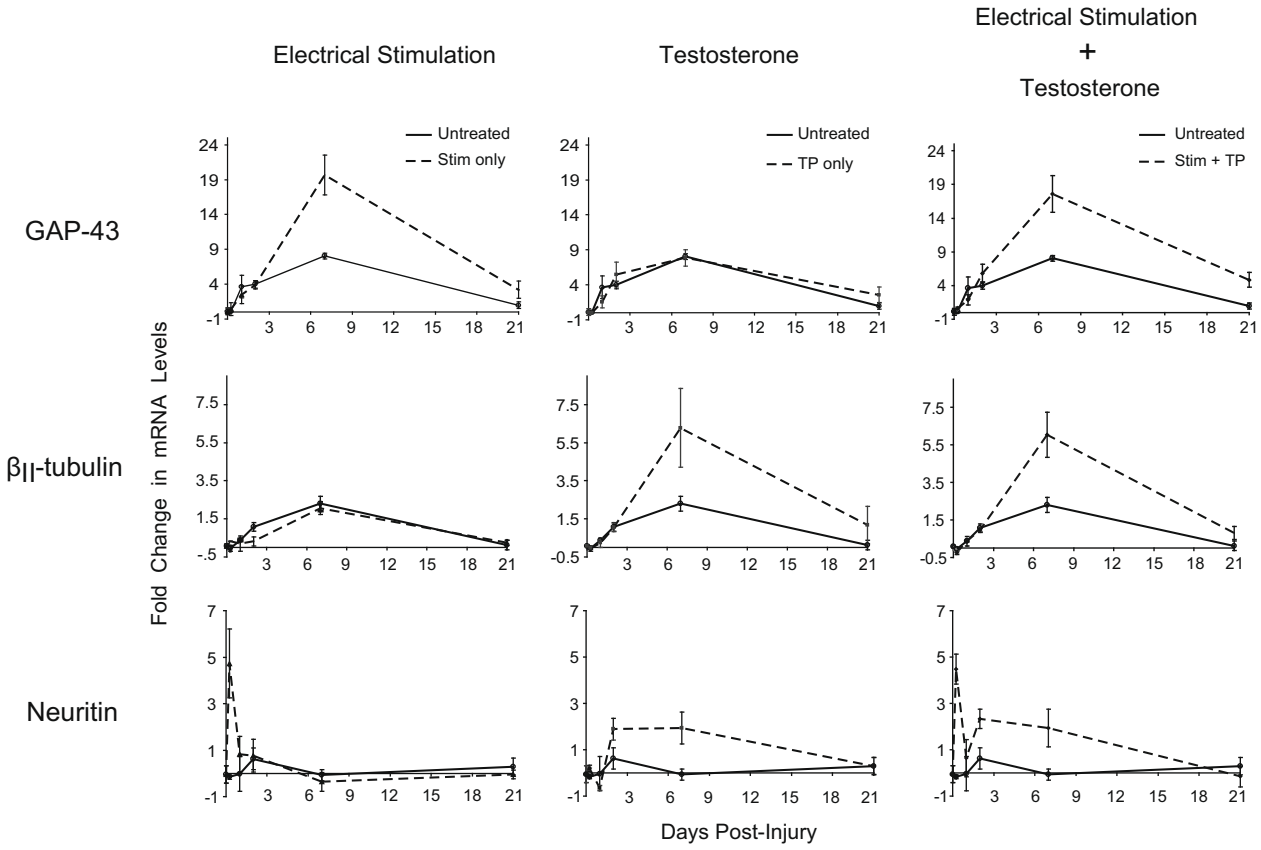

Fig. 5. Testosterone and electrical stimulation differentially regulate regeneration-associated gene expression in the facial motor nucleus following crush axotomy. Unilateral crush injuries were performed in male rats, and each animal was treated with electrical stimulation only, testosterone propionate only, or a combination of both electrical stimulation and testosterone propionate. Tissue punches were taken from the facial motor nucleus on both the injured and uninjured sides of each animal, at 6 h, 1 day, 2 days, 7 days, or 21 days after injury. mRNA levels were determined by reverse transcription and quantitative PCR, and are displayed as fold change with injury by comparing the injured and uninjured sides using the $\Delta\Delta C_t$ method. Changes in mRNA levels are shown for GAP-43 (top row), β_{II}-tubulin (middle row), and neuritin (bottom row), for untreated animals (solid lines, all panels), and animals treated with electrical stimulation only (left column), testosterone propionate only (center column), or a combination of both electrical stimulation and testosterone propionate (right column; all treated groups shown as dashed lines). GAP-43 was regulated by electrical stimulation only, β_{II}-tubulin was regulated by testosterone propionate only, and neuritin was regulated by both electrical stimulation and testosterone propionate. Points represent means ± SEM (data from [139]).

sparing from normally occurring neuron death through the enhancement of axonal and dendritic regeneration, androgens play an important role as protective and therapeutic agents in the nervous system. The accessibility of motor populations, the relative simplicity of their peripheral connectivity, and their clear behavioral role, combine to make them a powerful model in which to study processes of neuronal survival and regeneration. Androgens are profoundly effective in enhancing these processes, so exploring the neuroprotective effects of androgens on motoneurons has proven to be quite fruitful in recent decades. This approach has revealed a variety of mechanisms through which testosterone and its metabolites exert their protective effects on cellular morphology and function. Exploring these mechanisms and associated proteins and pathways will continue to provide valuable insights into regenerative processes, allowing us to identify specific treatment targets and establish new neurotherapeutic strategies.

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