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CRITICAL PERIOD FOR ESTROGEN-DEPENDENT MOTONEURON DENDRITE GROWTH IS COINCIDENT WITH ERα EXPRESSION IN TARGET MUSCULATURE

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Abstract

The spinal cord of rats contains the sexually dimorphic, steroid-sensitive motoneurons of the spinal nucleus of the bulbocavernosus (SNB). In males, SNB dendrite growth is dependent on gonadal steroids: dendrite growth is inhibited after castration, but supported in androgen- or estrogen-treated castrated males. Furthermore, estrogenic support of SNB dendrite growth is mediated by estrogen action at the target musculature, inhibited by estrogen receptor (ER) blockade at the muscle, and supported by local estradiol treatment However, this estrogenic support is restricted to the early postnatal period, after which the morphology of SNB dendrites is insensitive to estrogens. To test if the developmentally restricted effects of estrogens on SNB dendrite growth coincides with the transient expression of ER in the target musculature, ERα expression was assessed during development and in adulthood. ERα expression in extra-muscle fiber cells was greatest from postnatal day 7 (P7) to P14, and declined after P21. Because this pattern of ERα expression coincided with the period of estrogen-dependent dendrite growth, we tested if limiting hormone exposure to the period of maximal ERα expression in extra-muscle fiber cells could fully support estrogen-dependent SNB dendrite growth. We restricted estradiol treatment in castrated males from P7-P21, and assessed SNB dendritic morphology at P28. Treating castrates with estradiol implants at the muscle from P7-P21 supported dendrite growth to normal levels through P28. These data suggest that the transient ERα expression in target muscle could potentially define the critical period for estrogen-dependent dendrite growth in SNB motoneurons.

Indexing terms

gonadal hormones; motoneurons; dendrites; spinal cord; rat

INTRODUCTION

The lumbar spinal cord of rats contains a sexually dimorphic nucleus, the spinal nucleus of the bulbocavernosus (SNB), also known as the dorsomedial nucleus (DM; Schrøder, 1980). In male rats, the SNB consists of ~ 200 motoneurons that innervate the perineal muscles bulbocavernosus (BC), levator ani (LA) and the anal sphincter (Breedlove and Arnold, 1980; Schrøder, 1980; McKenna and Nadelhaft, 1986). The BC/LA muscles attach to the base of the penis, and are necessary for successful copulation and insemination (Sachs, 1982; Hart and Melese-D'Hospital, 1983). In female rats, the perineal musculature is absent or greatly reduced and the SNB consists of ~60 motoneurons that predominantly innervate

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the external anal sphincter (Hayes, 1965; Čihák et al., 1970; McKenna and Nadelhaft, 1986; Tobin and Joubert, 1991). The morphology of SNB motoneurons is also sexually dimorphic, and SNB somata of adult females are typically half as large as those of adult males (Breedlove and Arnold, 1981; McKenna and Nadelhaft, 1986).

The dimorphisms in the SNB neuromuscular system result from the actions of gonadal steroids, operating both developmentally and in adulthood During perinatal development, androgens are responsible for the prevention of normally occurring motoneuron death (Nordeen et al., 1985), somal growth (Lee et al., 1989; Goldstein et al., 1990; Goldstein and Sengelaub, 1992), perineal musculature retention (Čihák et al., 1970), and neuromuscular synapse elimination (Jordan et al., 1989; Jordan et al., 1989). Conversion of testosterone to estrogenic metabolites does not appear to be involved in this regulation, and treatment with estradiol has no effect on SNB motoneuron survival (Breedlove et al., 1982; Goldstein et al., 1990), somal growth (Breedlove et al., 1982; Goldstein and Sengelaub, 1994; Burke et al., 1997; Hebbeler et al., 2002), target muscle retention (Breedlove et al., 1982; Burke et al., 1997), or neuromuscular synapse elimination (Jordan et al., 1995).

Conversely, dendritic development in the SNB is regulated by both androgens and estrogens. During normal development, SNB dendrites grow profusely during the first 4 postnatal weeks (Goldstein et al., 1990). This growth is steroid-dependent: In males castrated 7 days after birth, dendrites fail to grow beyond their precastration lengths, whereas dendritic lengths of castrated males receiving testosterone replacement are not different from those of intact males by 4 weeks of age (Goldstein et al., 1990). Treatment with either of testosterone's metabolites, dihydrotestosterone or estradiol, partially supports dendritic growth (Goldstein and Sengelaub 1994), but combined treatment with both metabolites is as effective as testosterone in fully supporting dendritic growth (Goldstein and Sengelaub, 1994). Estrogenic support of postnatal dendritic growth appears to bemediated by estrogen action at the neuromuscular periphery, as local blockade of estrogen receptors (ERs) with tamoxifen implants at the SNB target musculature during the period of dendritic growth results in significantly decreased dendritic lengths, but placing identical implants at the scapula had no effect on SNB dendritic growth (Nowacek and Sengelaub, 2006). Conversely, treatment with estradiol muscle implants in castrates supports dendritic growth through postnatal day 28, demonstrating that estradiol action at the periphery during the early postnatal period is critical for SNB dendrite growth (Nowacek and Sengelaub, 2006).

This estrogenic support of SNB dendrites is transient, restricted to the early postnatal period: by the seventh week of age, dendritic lengths in estradiol-treated castrates are not different from those in oil-treated castrates, and are shorter than those of castrates treated with either testosterone or dihydrotestosterone (Goldstein and Sengelaub, 1994). In adulthood, SNB dendritic lengths in castrates can be fully maintained with dihydrotestosterone or testosterone, but estradiol is completely ineffective in supporting dendritic morphology (Verhovshek et al., 2010). Because estrogen-dependent SNB dendrite growth is transient and mediated by estrogen action at ERs in the target musculature, we hypothesized that the developmentally restricted estrogen-dependent SNB dendrite growth was mediated by a change in ER expression at the target musculature. In Experiment 1, we assessed ERα labeling in the BC muscle throughout the period of estrogen-dependent SNB dendrogenesis, and later in postnatal development and in adulthood, when SNB dendrites are insensitive to estrogens.

Experiment 1

MATERIALS AND METHODS

Animals—Untimed pregnant rat dams (Sprague-Dawley, Harlan, Indianapolis, IN) were maintained on a 12-h light, 12-h dark cycle, with unlimited access to food and water. When pregnant females gave birth (day of birth = P1), pups were sexed and litters culled when necessary, with preference for males over females. Males were killed weekly from P7 to P49, and in adulthood (approximately 100 d old); overall $n = 40$ (P7, $n = 6$; P14, $n = 6$; P21, $n = 6$; P28, $n = 6$; P35, $n = 4$; P42, $n = 3$; P49, $n = 3$; adult, $n = 6$). All procedures were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Bloomington Institutional Animal Care and Use Committee.

Immunohistochemistry—Animals were weighed, overdosed with urethane (0.5 ml/100 g of body weight), and perfused transcardially with saline followed by cold 4% paraformaldehyde in 0.1M phosphate buffer (pH = 7.4). The BC/LA muscles were removed and postfixed in the same fixative for 24 h and transferred to 30% sucrose in 0.1M phosphate buffer for a minimum of 24 h of cryoprotection. The BC/LA was cut horizontally into 12 μm sections on a cryostat at −16°C. Sections were thaw-mounted onto gelatin-coated slides, and stored at −16°C. For immunohistochemical processing, slides were brought to room temperature, removed from the slide boxes, placed in a glass staining rack and rinsed 3 × 5 min in phosphate buffered saline (PBS, pH 7.4). After rinsing, a hydrophobic border (Super Pap Pen, Ted Pella, Inc., Redding, CA) was created around each tissue section, and the first incubation did not occur until the border had dried (about 3 min). All incubations occurred in a humidified chamber and were performed by pipetting 200 μl of solution onto each section.

Sections were incubated for 45 min at room temperature in blocking solution containing 10% normal goat serum (NGS; Vector Laboratories, Inc., Burlingame, CA) and 0.2% Triton X-100 in PBS. Sections were then incubated for 48 h at 4°C in 4% NGS in PBS containing an antibody directed against the last 14 amino acids of the rat ERα(C1355, 1:1000; Millipore, Temecula, CA). After primary ERα incubation, sections were rinsed 3 × 5 times in PBS and incubated for 2 h at room temperature in 4% NGS in PBS containing a conjugated secondary antibody (goat anti-rabbit Alexa Fluor 488 $F(ab')_2$ fragments (H+L), 1:200; Invitrogen, Eugene, OR). Sections were then rinsed 3 × 5 min in PBS, and incubated in a 4% NGS in PBS solution containing a mouse monoclonal antibody for basal lamina (D18 supernatant, 1:50; Developmental Studies Hybridoma Bank, Iowa City, IA) for 12–18 h at 4°C. Following this incubation, sections were rinsed 3 × 5 times in PBS and incubated for 2 h at room temperature in a secondary antibody (goat anti-mouse IgG-TRITC, 1:200; Sigma-Aldrich, Oakville, Ontario, Canada). Sections were then rinsed 3 × 5 min in PBS, briefly air dried, and coverslipped with aqueous mounting fluid (Vectashield HardSet Mounting Medium; Vector Laboratories, Inc.). After slides dried for 5 min at room temperature, they were sealed with nail polish, dried for 10 min at room temperature, laid flat to dry overnight at 4°C, and then stored at 4°C. To control for nonspecific staining, control sections incubated without both primary antibodies were generated and demonstrated no immunostaining.

Microscopic Analysis—Because all of our previous developmental work on SNB motoneuron dendritic growth, hormone sensitivity, and critical period effects were done in BC-projecting motoneurons, our analysis was restricted to the BC muscle. After slides were brought to room temperature, sections of muscle were first viewed at 150–350X magnification under epifluorescent illumination to visualize basal lamina staining. The BC is a relatively complex muscle, with muscle fibers traveling in several different directions,

resulting in fields containing both cross- and longitudinally sectioned muscle fibers (Fargo et al., 2003); fields containing large numbers of basal lamina-stained muscle fibers in cross-section were selected for analysis. Because cross-sectional muscle fiber size varied across ages, variable magnifications and sampling frames were employed. After locating areas of tissue that contained sufficient numbers of cross-sectioned muscle fibers, magnification was increased until the field of view contained a minimum of 14 fibers, resulting in a final magnification of at least 1450X. Using Stereo Investigator (MBF Bioscience, Inc., Williston, VT), muscle fibers were sampled systematically, by either analyzing all fibers in a field of view or superimposing a grid on the tissue image (e.g., 100 μm x 100 μm square frame) and sampling all fibers within the frame.

To quantify ERα immunostaining, muscle fibers were visualized under FITC fluorescence and images were captured for subsequent analysis. Areas that appeared to be labeled relative to background (see below) were identified as either in-fiber or extra-fiber depending on the location of ERα label relative to the basal lamina stain. One field per section and 1–3 sections per animal were analyzed; overall, an average 69.40 muscle fibers per animal were traced. For each analyzed tissue section, the mean and standard deviation of muscle fiber FITC luminance values were calculated and a minimum labeling criterion of the mean plus two standard deviations was established for each section. Cells that exceeded this criterion were identified as ERα-positive and were categorized as either ERα-positive muscle fibers (if surrounded by a basal lamina) or ERα-positive extra-muscle fiber cells. Because muscle fiber area increases with age (potentially decreasing the density of immunostained muscle fibers), we sampled equivalent numbers of muscle fibers across ages and expressed ERα-positive muscle fibers as a percentage of the sample. ERα-positive extra-muscle fiber cells were identified only on the basis of ERα labeling, so these numbers were expressed as a density per unit area. The percentage of ERα-positive muscle fibers and density of ERα-positive extra-muscle fiber cells per section were analyzed using one-way ANOVAs with Fisher's LSD post-hoc tests. Differences were considered significant if $p < 0.05$.

RESULTS

ERα immunolabeling was present in the BC muscle at all ages examined. Immunolabeling in muscle fibers was not restricted to nuclei, suggesting cytosolic localization. ERα immunolabeling was categorized as either ERα-positive muscle fibers (label contained within the basal lamina stain) or ERα-positive extra-muscle fiber cells (label outside basal lamina stain; Fig. 1). The average number of muscle fibers analyzed per animal did not differ across ages [$F(7, 32) = 1.21$, n.s.]. For each animal, the number of labeled muscle fibers was expressed as a percentage of total muscle fibers sampled. The percentage of ERα-positive muscle fibers did not vary with age [mean = 7.01% ± 0.89; $F(7, 32) = 2.20$, n.s.].

There was a significant main effect of age on the density of ERα-positive extra-muscle fiber cells [$F(7, 32) = 2.32$, $p < 0.05$; Fig. 2]. ERα-positive extra-muscle fiber cells were abundant at P7 (209.3 ± 102.8 per mm^2) and P14 (285.2 ± 115.1 per mm^2) compared to adult levels (LSDs, ps < 0.05). At P21, the density of ERα-positive extra-muscle fiber cells was decreased by 38%, and continued to decline into adulthood (0.00 ± 0.0 per mm^2). The densities of ERα-positive extra-muscle fiber cells after P21 did not differ significantly from adult levels (LSDs, n.s.).

Experiment 2

In Experiment 1, we found that the density of ERα-positive BC extra-muscle fiber cells decreased significantly after P21. Based on these results, we hypothesized that BC extra-muscle fiber cells are the site of estrogenic action in supporting SNB dendrite growth, and that the transient expression of ERα within BC extra-muscle fiber cells during the early

postnatal period could define the critical period for estrogen-dependent SNB dendrite growth. To test this hypothesis, in Experiment 2, we castrated males and treated them with estradiol via BC muscle implants from P7 to P21, and assessed SNB dendritic morphology at P28, the day at which SNB dendrites normally reach maximal length (Goldstein et al., 1990).

MATERIALS AND METHODS

Animals—Untimed pregnant rat dams (Sprague-Dawley; Harlan) were maintained on a 12-h light, 12-h dark cycle, with free access to food and water. Litters were culled to eight pups when necessary, retaining males preferentially. Males were bilaterally castrated under isoflurane anesthesia at P7, and were given estradiol implants (see below) at the left BC muscle or were untreated ($n = 7$). In estradiol-treated castrated males, some implants were removed at P21 ($n = 6$), and in other animals, implants remained until P28 ($n = 5$). Age-matched intact control males ($n = 5$) were also used (overall $n = 23$). All procedures were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the Bloomington Institutional Animal Care and Use Committee.

Implants—To restrict estradiol treatment to the target musculature, we used our previous method (Nowacek and Sengelaub, 2006) to deliver hormones locally to the BC muscle. This treatment produces alterations in motoneuron morphology that can be directly ascribed to local rather than systemic effects; furthermore, the treatment has no effect on the normal development of either the BC muscle or SNB motoneurons, and no effect on dendritic labeling or tracer transport (Nowacek and Sengelaub, 2006). Small (0.85 mm^3) implants impregnated with estradiol (Steraloids, Newport, RI) were constructed by mixing crystalline estradiol with Silastic adhesive (Dow-Corning, Auburn, MI) and compressing the mixture between two glass slides (lightly greased with petroleum jelly) separated by a spacer (0.85 mm thick), which was allowed to cure. The cured Silastic was cut into 1 × 1 mm pieces (total volume 0.85 mm^3) and coated on five sides with acrylic. The amount of estradiol in each implant (0.1 mg) was identical to that used previously; this dosage does not produce systemic effects, and supports SNB dendritic growth.

Histochemistry—Horseradish peroxidase conjugated to the cholera toxin β subunit (BHRP; List Biological, Campbell, CA) was used to retrogradely label SNB motoneurons innervating the BC muscle. Previous studies have demonstrated that BHRP labeling permits sensitive detection and quantitative analysis of SNB somal and dendritic morphologies (Kurz et al., 1986; Goldstein and Sengelaub, 1990; Hebbeler and Sengelaub, 2003). SNB motoneuron morphology was examined at P28 (when SNB dendritic length is normally maximal; Goldstein et al., 1990). At P26, animals were anesthetized with isoflurane, the perineal muscles exposed, and BHRP unilaterally injected (0.5 μl; 0.2% solution) into the left BC muscle, the same muscle for those males that received muscle implants. Forty-eight h after BHRP injection, a period that ensures optimal labeling of SNB motoneurons (Kurz et al., 1986; Goldstein et al., 1990), animals were overdosed with urethane (0.5 ml/100 g body weight) and perfused intracardially with saline followed by cold 1% paraformaldehyde/ 1.25% glutaraldehyde fixative. Lumbar cords from all animals were removed, postfixed in the same fixative for 5 h, and transferred to sucrose phosphate buffer (10% w/v, pH = 7.4) overnight for cryoprotection. Spinal cords were then embedded in gelatin, frozen, and sectioned transversely at 40 μm; all sections were collected into four alternate series. For visualization of BHRP, the tissue was immediately reacted using a modified tetramethyl benzidine protocol (TMB; Mesulam, 1982), mounted on gelatin-coated slides, counterstained with thionin, and cover-slipped with Permount. BC/LA muscles were removed at perfusion and weighed to evaluate potential treatment effects on gross muscle development.

Motoneuron Somata—The number of BHRP-filled motoneurons was assessed in all sections through the entire rostrocaudal extent of the SNB for all animals. Counts of labeled motoneurons in the SNB were made under brightfield illumination, where somata and nuclei could be visualized and cytoplasmic inclusion of BHRP reaction product confirmed. Estimates of the total number of labeled SNB motoneurons were obtained using the optical dissector method outlined by Coggeshall (1992) and a procedure similar to that of West and Gunderson (1990). This method yields an unbiased count of SNB motoneurons (Raouf et al., 2000). Counts were made at 500X; motoneuron somata could be easily visualized in multiple focal planes. BHRP-labeled motoneurons were counted as their somata first appeared in focus while focusing through the *z* axis, and labeled somata in the first focal plane (i.e., "tops") were not counted. For each animal, counts were derived from sections spaced at 160 μm intervals uniformly distributed through the entire rostrocaudal extent of the SNB. Within each section, all labeled somata within the SNB were counted. Estimates of the total number of labeled SNB motoneurons were then obtained by correcting for percentage of the tissue sampled.

The cross-sectional soma area of BHRP-labeled motoneurons was measured in an average of 21.8 motoneurons for each animal using a video-based morphometry system (Stereo Investigator; MBF Bioscience, Inc.) at a final magnification of 1350X. Soma areas within each animal were averaged for statistical analysis. The optical density of labeled somata was also measured under brightfield illumination to confirm equivalence of BHRP labeling density.

Dendritic Length—For each animal, dendritic lengths in a single representative set of alternate sections were measured under darkfield illumination. Beginning with the first section in which BHRP-labeled fibers were present, labeling through the entire rostrocaudal extent of the SNB dendritic field was assessed in every other section (320 μm apart) in three dimensions using a computer-based morphometry system (Neurolucida; MBF Bioscience, Inc.; final magnification 250X) to yield both composite illustrations of the arbor and measurements of individual fiber lengths. All BHRP-labeled fibers were drawn regardless of location, size, or contiguity with labeled cell bodies to ensure a complete assessment of dendritic length. Because the entire rostrocaudal range of the SNB dendritic field in each animal was sampled, this method allows for a complete assessment of SNB dendrites in both the transverse and horizontal planes. Average dendritic arbor per labeled motoneuron was estimated by summing the measured dendritic lengths of the series of sections, multiplying by two to correct for sampling, then dividing by the total number of labeled motoneurons in that series. This method does not attempt to assess the actual total dendritic length of labeled motoneurons (Kurz et al., 1991), but has been shown to be a sensitive and reliable indicator of changes in dendritic morphology in normal development (Goldstein et al., 1990), after hormonal or surgical manipulation (Kurz et al., 1986; Goldstein et al., 1990; Kurz et al., 1991; Goldstein and Sengelaub, 1994; Goldstein et al., 1996; Hays et al., 1996; Hebbeler and Sengelaub, 2003), due to dendritic interactions (Goldstein et al., 1993), or after NMDA receptor blockade (Hebbeler et al., 2002).

To assess potential redistributions of dendrites across treatment groups, for each animal the composite dendritic arbor created in the length analysis was divided using a set of axes radially oriented around the central canal, dividing the spinal cord into 12 bins of 30° each. The portion of each animal's dendritic arbor per labeled motoneuron contained within each location was then determined.

Dendritic Extent—The comparability of BHRP labeling across groups was assessed by quantifying both the rostrocaudal and radial dendritic extent of SNB arbors. The rostrocaudal extent of the dendritic arbor was determined by recording the distance between

the first and last section in which labeled SNB dendrites were present for each animal. In the transverse plane, for each animal the maximal radial extent of the dendritic arbor for each section throughout the rostrocaudal extent of the SNB dendritic field was measured using the same set of axes and 30° bins used for the dendritic distribution analysis. For each bin, the distance between the central canal and the most distal BHRP-filled process was measured.

Statistical analysis consisted of analyses of variance (one- or two-way with repeated measures) followed by appropriate planned comparisons (Fisher's Protected LSD) as described below. Digital light micrographs were obtained using an MDS 290 digital camera system (Eastman Kodak Company, Rochester, NY). Brightness and contrast of these images were adjusted in Adobe PhotoShop (Adobe Systems, Inc., San Jose, CA).

RESULTS

BC/LA Muscle Weight—BC/LA muscle weights did not differ across groups [$F(3,16) = 1.87$, n.s.; intact control males = 0.091 ± 0.01 g, castrates = 0.094 ± 0.01 g, castrates treated with estradiol implants from P7-P21 = 0.073 ± 0.02 g, castrates treated with estradiol implants from P7-P28 = 0.077 ± 0.02 g].

Morphometry—Injections of BHRP into the left BC successfully labeled ipsilateral SNB motoneurons in all animals in a manner consistent with previous studies (Kurz et al., 1986; Goldstein and Sengelaub, 1990; Goldstein and Sengelaub, 1994; Burke et al., 1997; Burke et al., 1999). SNB motoneurons displayed their characteristic multipolar morphologies, with dendritic arbors projecting ventrolaterally, dorsomedially, and across the midline into the area of the contralateral SNB (Fig. 3). The number of BHRP-labeled SNB motoneurons (51.30 ± 4.64) did not differ across groups [$F(3,19) = 2.39$, n.s.].

Soma Area—The size of SNB somata at P28 differed significantly across groups [$F(3,19) = 6.63$, $p < 0.05$; Fig. 4]. The mean cross-sectional area of SNB somata in intact control males (837.77 ± 12.55 μm^2) was typical. The somata of untreated castrates (661.15 ± 21.11 μm^2, reduced 21%), or castrates treated with estradiol implants from P7-P21 (756.31 ± 101.4 μm^2, reduced 17%) or P7-P28 (647.26 ± 41.89 μm^2, reduced 23%) were all significantly smaller than those of intact control males (LSDs, $p < 0.05$).

Dendritic Length—The overall length of SNB dendrites significantly differed across groups [$F(3,19) = 17.02$, $p < 0.05$; Fig. 5]. SNB dendritic length in intact control males was typical (5963.02 ± 473.75 μm), reflecting the exuberant growth that occurs over the first four postnatal weeks. Dendritic lengths in castrates were approximately 67% shorter (1943.61 ± 604.17 μm) than those of intact control males at P28 (LSD, $p < 0.05$). Treatment of castrates with estradiol implants from P7-P21 or P7-P28 resulted in dendritic lengths (8237.88 ± 1146.67 μm, 8198.10 ± 417.01 μm, respectively) that were not different from those of intact control P28 males (LSDs, n.s.).

Dendritic Distribution—The SNB dendritic arbor of intact control males is radially organized but not uniformly distributed, with over 50% of the arbor concentrated ventrolaterally between 180 and 300° (Goldstein et al., 1993). The distribution of SNB dendrites showed an effect of group [repeated measures $F(3,209) = 17.39$, $p < 0.05$], as well as the typical significant effect of location [repeated measures $F(11,209) = 80.48$, $p < 0.05$; Fig. 6], and a group by location interaction [$F(33,209) = 6.971$, $p < 0.05$]. Castration not only reduced overall dendritic length, but it did so throughout the arbor, resulting in amounts of dendritic material per bin ranging from 26 to 42% of intact control male values [$F(3,19) = 17.02$, $p < 0.05$]. Castrates treated with estradiol implants from P7-P21 displayed a dendritic

distribution that differed from that of intact control males [group by location interaction, $F(11,99) = 44.80$, $p < 0.05$]. This interaction was the result of an altered dendritic distribution in P7-P21 estradiol-treated castrates ventrolaterally between 210 and 240 degrees, where the amount of dendritic arbor was increased by 155% [$F(1,9) = 5.78$, $p < 0.05$]. Similarly, treating castrates with estradiol implants from P7-P28 resulted in an altered dendritic distribution, [group by location interaction, $F(11,88) = 2.60$, $p < 0.05$]. Again, this interaction was the result of an altered dendritic distribution in P7-P28 estradiol-treated castrates ventrolaterally between 210–240 degrees [$F(1,8) = 10.78$, $p < 0.05$], where the amount of dendritic arbor was enhanced by 173%.

Dendritic Extent—The distance spanned by SNB dendrites in the rostrocaudal axis did not differ across treatment groups [$F(3,19) = 1.22$, n.s.; intact control males = 2432.00 ± 283.52 μm, castrates = 2377.14 ± 161.62 μm, castrates treated with estradiol from P7-P21 = 2693.33 ± 96.15 μm, castrates treated with estradiol from P7-P28 = 2752.00 ± 93.30 m]. The radial extent of BHRP labeling showed an effect of group [$F(3,19) = 17.02$, $p < 0.05$; Fig. 7]. The effect of group was due to decreases in radial extent in castrates; radial extent did not differ between intact control males and estradiol-treated castrates [$F(2,13) = 0.17$, n.s.].

DISCUSSION

Immunolabeling for ERα in the BC muscle revealed the presence of ERα during development as well as in adulthood. The percentage of ERα-positive muscle fibers in the BC muscle did not change with age. In contrast, the density of ERα-positive extra-muscle fiber cells changed dramatically and was greatest during the early postnatal period, when estrogens act at the musculature to facilitate SNB dendritic growth, but absent in adulthood, when SNB motoneurons are insensitive to estrogens. The current results suggest that ERα located within these extra-muscle fiber cells could be the site of action for estrogenic support of SNB motoneuron dendrite growth during the first four weeks of postnatal development in male rats.

ERα Immunolabeling

Binding of estrogens by the BC/LA muscle has been previously reported (Dube et al., 1976), so the presence of ERs in this muscle was expected. However, our results describe a specific isoform of the receptor — ERα — and its expression in this tissue, and further demonstrate that the BC's ability to bind estrogens is present both throughout development and in adulthood. Interestingly, ERα immunostaining did not appear to be restricted to the nuclei of muscle fibers. This is consistent with previous reports of the localization of ERα in skeletal muscle, indicating the presence of ERα in cytosol, and in association with particular subcellular fractions (e.g., mitochondrial; Milanesi et al., 2008).

Our results demonstrate that the percentage of ERα-positive muscle fibers did not differ across development or in adulthood. Because BC muscle fiber number is stable across the time points used in this study (Tobin and Joubert, 1991), measurements of ERα-positive muscle fibers are not biased by an alteration in total muscle fiber number. Furthermore, since there is a change in estrogen's effects on dendrites from development to adulthood, but the proportions of ERα-positive muscle fibers do not change during these time periods, it is unlikely that ERα expression within muscle fibers mediates these transient estrogenic effects on SNB dendrites. It is possible that the other predominant ER isoform — ERβ — could mediate the estrogenic effects on SNB dendrites via the BC. Therefore, not observing a change in ERα expression in muscle fibers does not rule out a role of muscle fibers in the estrogenic support of motoneuron dendrite growth during the early postnatal period.

However, it is also possible that ERβ is not expressed in BC muscle, because there is great variability in the differential expression of ERα and ERβ depending on the target tissue and many other factors (Banie et al., 2008).

Although ERα expression in muscle fibers did not change across ages, the density of ERα-positive extra-muscle fiber cells was significantly different during early postnatal development compared to adult animals. We found that the density of ERα-positive extra-muscle fiber cells was significantly higher in P7 and P14 animals compared to adults. These results demonstrate that ERα is present in BC extra-muscle fiber cells during the time when estrogens act at this muscle to promote SNB dendritic growth. The density of ERα-positive extra-muscle fiber cells is substantially lower or zero at later ages, when SNB motoneurons are insensitive to estrogens. Since the expression of ERα in extra-muscle fiber cells coincides with the critical period for estrogen action at the muscle on SNB dendritic growth, this suggests that these cells could be the site of action for estrogen-mediated SNB dendritic growth during the early postnatal period.

Previous work has demonstrated that the robust growth of SNB dendrites during the first four postnatal weeks of life is steroid-dependent (Goldstein et al., 1990). These studies all used treatment periods lasting through P28. Based on the changes in density of ERα-positive BC extra-muscle fiber cells we hypothesized that the critical period for estradiol exposure in supporting dendritic growth ended earlier than P28. Our results demonstrate that the period of high density of ERα-positive BC extra-muscle fiber cells may define the critical period for estrogen-dependent SNB dendrite growth during the early postnatal period: treating castrates with estradiol implants at the BC muscle when the density of ERα-positive extra-muscle fiber cells is high fully supports SNB dendrite growth, resulting in dendrite lengths that are not different from those of P28 intact control males or animals treated from P7-P28 with estradiol implants at the BC.

Target Musculature

BC/LA muscle development is androgen-dependent (Čihák et al., 1970; Tobin and Joubert, 1991). In males, the number of myotubes, mononucleate cells, and muscle fibers in the BC/LA increases during the first week of life, and exposure to androgens during the first week of life is sufficient to support BC/LA growth through P28 (Hebbeler and Sengelaub, 2003; Tobin and Joubert, 1991; present study). Furthermore, because BC/LA weights did not differ, any difference in SNB morphology could not be due to differences in muscle size.

Motoneuron Morphology

Somata—In normal males most SNB somal growth occurs during the first four postnatal weeks, and this increase in soma size is androgen-dependent (Breedlove and Arnold, 1983; Breedlove and Arnold, 1983; Goldstein and Sengelaub, 1990; Goldstein and Sengelaub, 1994). In the present study, SNB somata in castrates were smaller than those of intact control males, consistent with previous findings demonstrating the androgen-dependence of SNB soma growth. Treatment of castrates with estradiol (from P7-P21 or P7-P28) did not support SNB somal growth during the first four postnatal weeks, consistent with previous work demonstrating that estradiol fails to masculinize SNB soma size (Breedlove et al., 1982; Goldstein and Sengelaub, 1994; Burke et al., 1997; Hebbeler et al., 2002; Nowacek and Sengelaub, 2006) and blocking synthesis of estrogens with aromatase inhibition does not interfere with the development of masculine soma size (Burke et al., 1999).

Dendrites—Our results demonstrate that castration results in significantly reduced SNB dendrite lengths, replicating previous findings (Goldstein et al., 1990; Goldstein and Sengelaub, 1994; Nowacek and Sengelaub, 2006). Furthermore, these results confirm the

neuromuscular periphery as the site of action for estrogen-dependent SNB dendrite growth during the early postnatal period (Nowacek and Sengelaub, 2006). ERs are found in non-muscle fiber cells, including terminal Schwann cells (Jung-Testas et al., 1993). In the current study, we demonstrated that ERα expression in a BC extra-muscle fiber cell type coincides with the early postnatal period during which estradiol acts at the target musculature to promote SNB dendrite growth. Together, these data suggested that ERα expression in the BC muscle could be the site of action for mediating the critical period for estrogen-dependent SNB dendrite growth during the early postnatal period. In the present study, we restricted estradiol treatment at the BC muscle to the period when the density of ERα-positive extra-muscle fiber cells was highest (P7-P21). Our results support the hypothesis that the period of estrogen-dependent SNB dendrite growth is potentially defined by the presence of ERα in extra-muscle fiber cells in the BC, as animals treated with estradiol implants from P7-P21 had dendritic arbors that were not different than intact control animals or castrates treated with estradiol implants from P7-P28.

Previous work has demonstrated the importance of the target musculature in the regulation of estradiol-dependent SNB dendrite growth. Whereas estrogens are important during early postnatal development for SNB dendritic growth, SNB motoneurons do not accumulate estradiol during development (Taylor et al., 1995) or in adulthood (Breedlove and Arnold, 1980; Breedlove and Arnold, 1983), suggesting that these hormones exert their effect of SNB dendritic growth indirectly. Neither primary sensory nor supraspinal afferents appear to be involved in SNB dendritic growth (Goldstein et al., 1996; Hays et al., 1996; Hebbeler and Sengelaub, 2003), and it has been suggested that the growth and differentiation of dendrites may be initiated and shaped by contact with the target tissues (Sumner and Watson, 1971; Yawo, 1987; Voyvodic, 1989). Furthermore, blocking androgen receptors at the BC muscle in adulthood results in a reduction of SNB dendritic lengths, demonstrating that hormone receptors in target tissues are critical for the target-mediated hormonal regulation of SNB morphology (Rand and Breedlove, 1995). Our data provide further evidence that estradiol-dependent SNB dendrite growth is regulated at the target musculature, and indicate that hormone receptor expression in BC muscles mediates this target-dependent dendrogenesis.

Similarly, our results indicate that hormone receptor expression in the target musculature can regulate motoneuron dendrite morphology. Recent work has demonstrated that there is a causal relationship between androgen receptor expression in the target musculature and degree of androgen sensitivity exhibited by the innervating motoneurons (Huguenard et al., 2011). By manipulating the expression of androgen receptors exclusively in the target musculature, androgen sensitivity was induced in a population of normally androgen-insensitive motoneuron dendrites, demonstrating that androgen sensitivity is directly conferred by receptor expression in the target musculature (Huguenard et al., 2011). Along with our findings, these results further implicate the importance of receptor expression in the target muscles for hormonal regulation of motoneuron morphology.

When examining the differences in dendritic distribution across groups, we found that treatment of castrates with estradiol at the BC muscle (either from P7-P21 or P7-P28) resulted in significantly longer SNB dendrites in the gray matter ventrolateral to the central canal compared to intact control animals. This area of SNB dendritic arbor where estrogen treatment promotes enhanced dendritic growth compared to intact control males has previously been identified as an important source of excitatory drive to the SNB (Tanaka and Arnold, 1993), and shows specific reductions in dendritic length following spinal transection early in development (Hebbeler and Sengelaub, 2003). Future research should determine why estradiol treatment at the BC muscle in castrates drives SNB dendritic development in this area of the arbor that is specialized for receiving excitatory inputs.

Comparability of BHRP labeling

Previous studies have demonstrated that neither axonal transport of BHRP (Leslie et al., 1991) nor dendritic transport as demonstrated by the rostrocaudal or mediolateral extent of dendritic labeling (Kurz et al., 1990; Goldstein and Sengelaub, 1994) is affected by hormone levels. In the present study, the possibility that hormone manipulations could affect retrograde transport is an important consideration, as such artifact could potentially result in apparent alterations in dendritic morphology. No difference in rostrocaudal extents of SNB dendrites were observed, but radial extents in untreated castrates were smaller than those of intact control males. This result most likely reflects the attenuated growth of SNB dendrites rather than a transport artifact, which (because rostrocaudal extent was not affected) would necessarily have had to occur selectively in the transverse plane. In contrast, no differences in either rostrocaudal or radial extents of dendrites were observed between intact control males and estradiol-treated castrates, indicating that the ability of SNB dendrites to transport BHRP out to the most distal, highest order branches was not affected in these groups. Thus, we believe the dendritic labeling across groups was comparable, allowing direct comparisons of dendritic length and distribution across groups.

Mechanisms of target-mediated dendritic growth

Specific features of the SNB system, including perineal muscle development and motoneuron morphology, are regulated by testosterone action at androgen receptors during a perinatal critical period (Freeman et al., 1996). To test the hypothesis that androgen receptors in BC/LA muscle fibers are directly responsible for testosterone-mediated masculinization of the SNB system, Niel et al. (2009) created a transgenic animal that expressed androgen receptors only in muscle fibers, but not other cell types. Despite the expression of androgen receptors, SNB motoneurons and the LA muscle exhibited the female phenotype in these animals, demonstrating that androgen receptor expression within muscle fibers was not sufficient to mediate sexual differentiation in this system. These authors suggested, as we do, that it is the expression of hormone receptors in a non-muscle fiber cell type in the BC/LA that is responsible for hormone effects on the SNB neuromuscular system (Niel et al., 2009).

These extra-muscle fiber cells could be fibroblasts, adipocytes, endothelial cells, epithelial cells, or Schwann cells, and it could be a change in ER in these cells or a change in the amount of these cells themselves, that mediates the change in SNB estrogen sensitivity from the early postnatal period to adulthood. While it is unclear how estradiol action at extra-muscle fiber ERα promotes SNB dendrite growth, a possible mechanism of action could be through the regulation of growth factors. For example, in adulthood, androgens regulate brain-derived neurotrophic factor (BDNF) expression in the target musculature, and BDNF can reduce SNB dendritic length (Verhovshek et al., 2010; Verhovshek and Sengelaub, 2010). Similarly, the ERα-positive extra-muscle fiber cells present postnatally could be supporting dendritic growth by suppressing BDNF expression during the early postnatal period. Future studies are needed to determine the identity of the extra-muscle fiber cells and their role during development.

In conclusion, the current data suggest that changes in ERα expression in the BC could define the critical period in which estrogens act at the target musculature to regulate SNB dendritic growth during the early postnatal period. Why this expression in extra-muscle fiber cells is limited to the postnatal period, and the mechanism by which it supports SNB dendrite growth remains to be determined.

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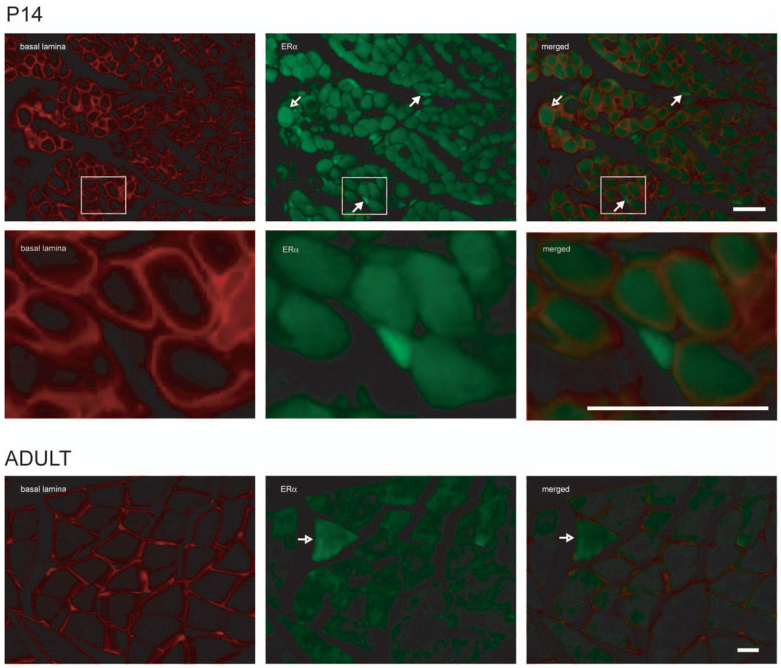

Figure 1.
Digital light micrographs of cross-sections of BC muscle in P14 (top row) with higher magnification views of indicated areas (middle row), and adult males (bottom row). Each row shows the same tissue section viewed under different epifluorescent illumination, demonstrating immunolabeling for basal lamina only (red; left), ERα only (green; middle), and the merged image showing combined basal lamina and ERα immunolabeling (right). Arrows indicate ERα-positive muscle fibers (open arrows) and ERα-positive extra-muscle fiber cells (white arrows); note the absence of basal lamina staining of the indicated extra-muscle fiber cell in the middle row. Scale bars = 25 μm.

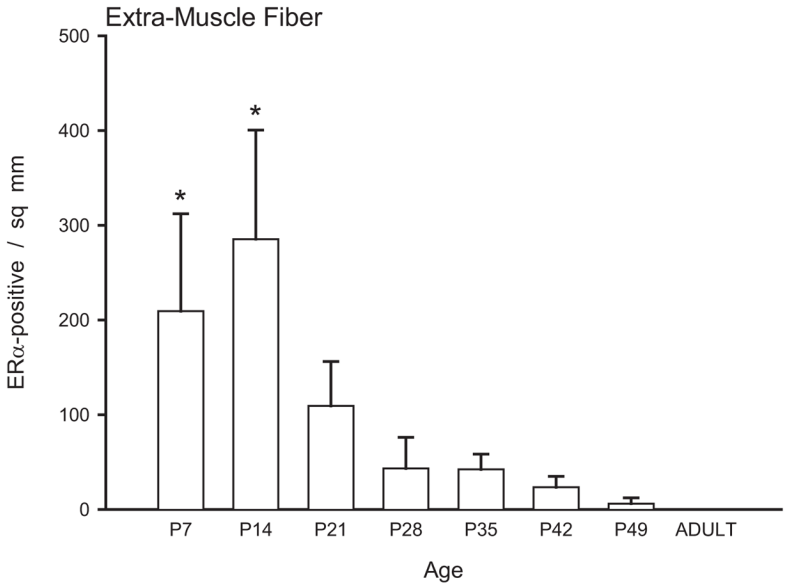

Figure 2.

The number of ERα-positive extra-muscle fiber cells per mm^2 in the BC at P7, P14, P21, P28, P35, P42, 49, and in adulthood. Bar heights represent means ± SEM for three to six animals per group. Asterisk denotes significantly different from adult animals.

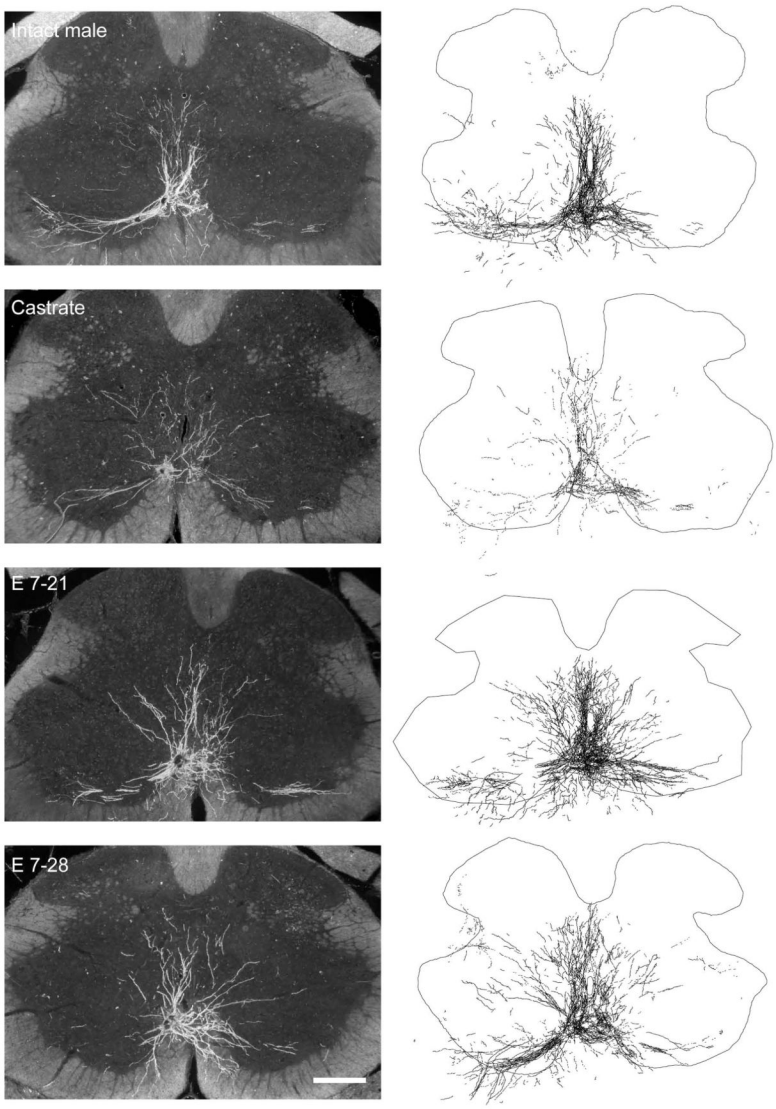

Figure 3.

(Left) Darkfield digital micrographs of transverse sections through the lumbar spinal cord of an intact male (top), an untreated castrate (second from top), a castrate treated with an estradiol implant from P7-P21 (E7–21; second from bottom), and a castrate treated with estradiol from P7-P28 (E 7–28; bottom) after BHRP injection into the left BC muscle at P28. (Right) Computer-generated composites of BHRP-labeled somata and processes drawn at 320 μm intervals through the entire rostrocaudal extent of the SNB; these composites were selected as they are representative of their respective group average dendritic lengths. Scale bar = 250 μm.

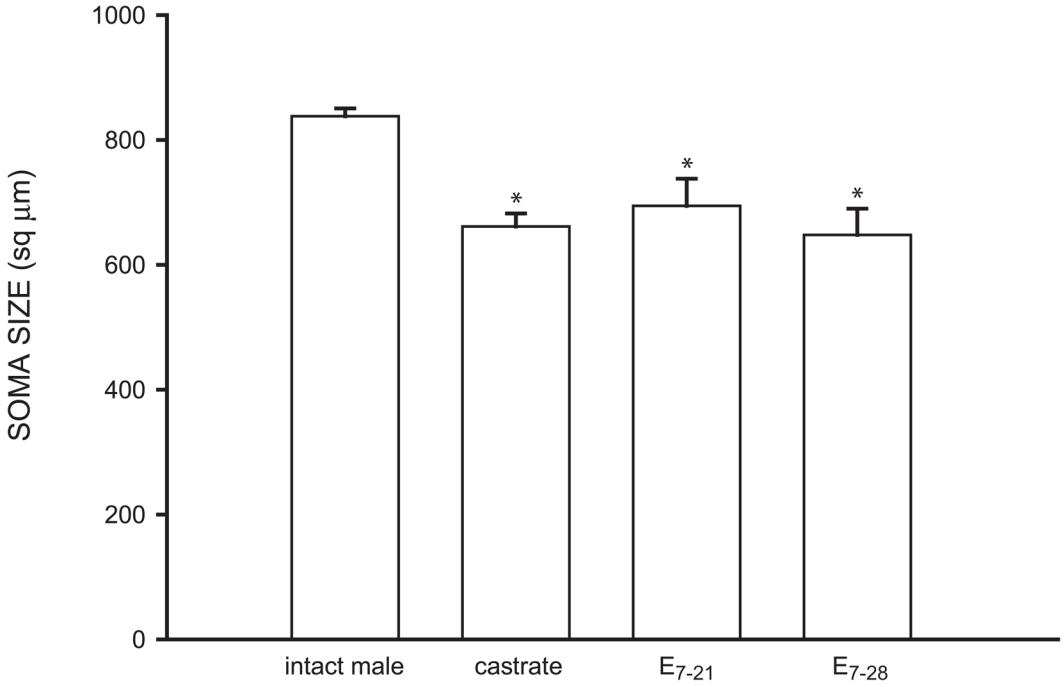

Figure 4.
Soma areas of SNB motoneurons at P28 for intact males, untreated castrates (castrate), and castrates with estradiol implants from P7-P21 ($E_{7–21}$) or from P7-P28 ($E_{7–28}$). Bar heights represent means ± SEM for four to six animals per group. Asterisk denotes significantly different from intact control males.

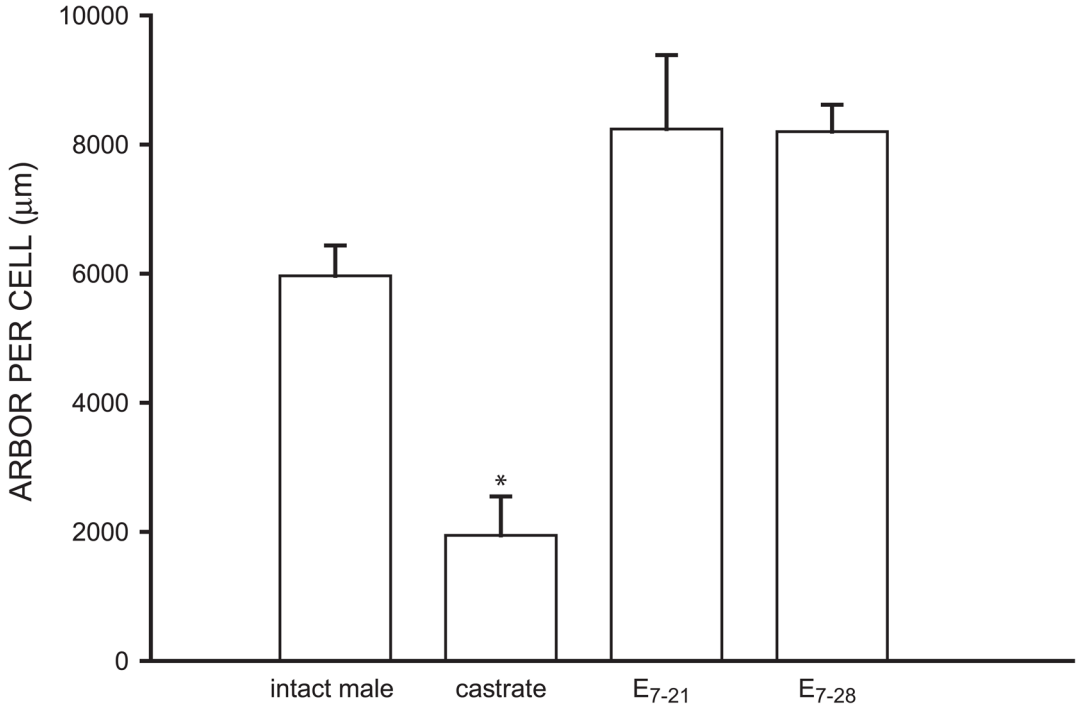

Figure 5.
Dendritic lengths expressed as length of arbor per labeled SNB motoneuron at P28 for intact males, untreated castrates (castrate), and castrates with estradiol implants from P7-P21 ($E_{7–21}$) or from P7-P28 ($E_{7–28}$). Bar heights represent means ± SEM for four to six animals per group. Asterisk denotes significantly different from intact control males.

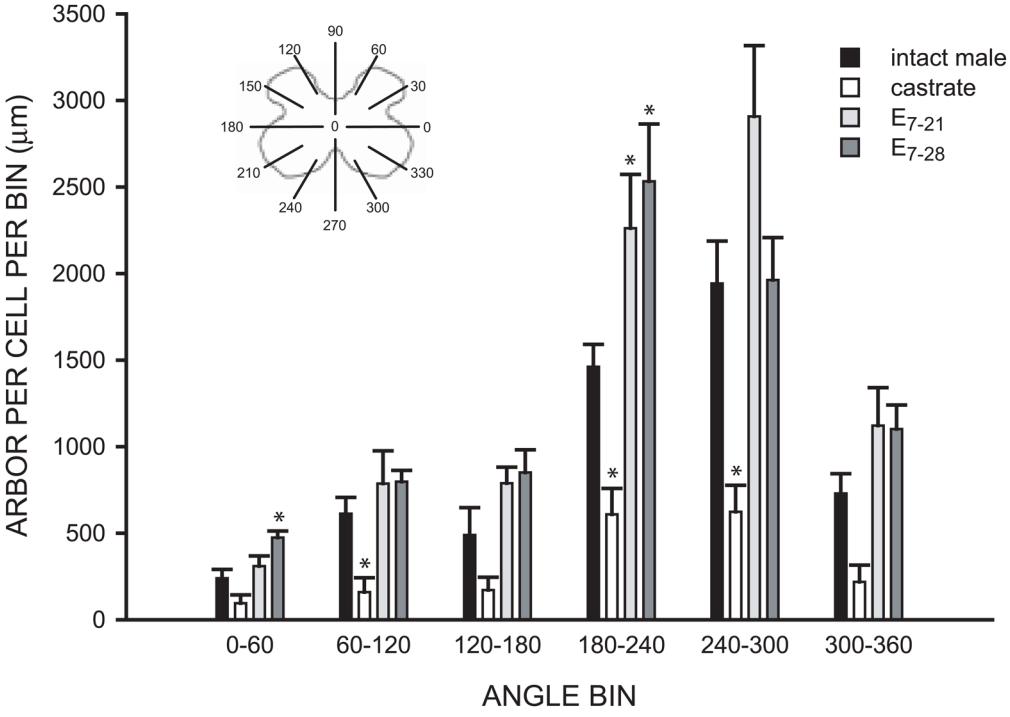

Figure 6.

(Top) Schematic drawing of spinal gray matter divided into radial sectors for measure of SNB dendritic distribution. (Bottom) Length per radial bin of SNB dendrites at P28 for intact males, untreated castrates (castrate), and castrates with estradiol implants from P7-P21 ($E_{7–21}$) or from P7-P28 ($E_{7–28}$). For graphical purposes, dendritic length measures have been collapsed into six bins of 60° each. Bar heights represent means ± SEM for four to six animals per group. Asterisk denotes significantly different from intact control males.

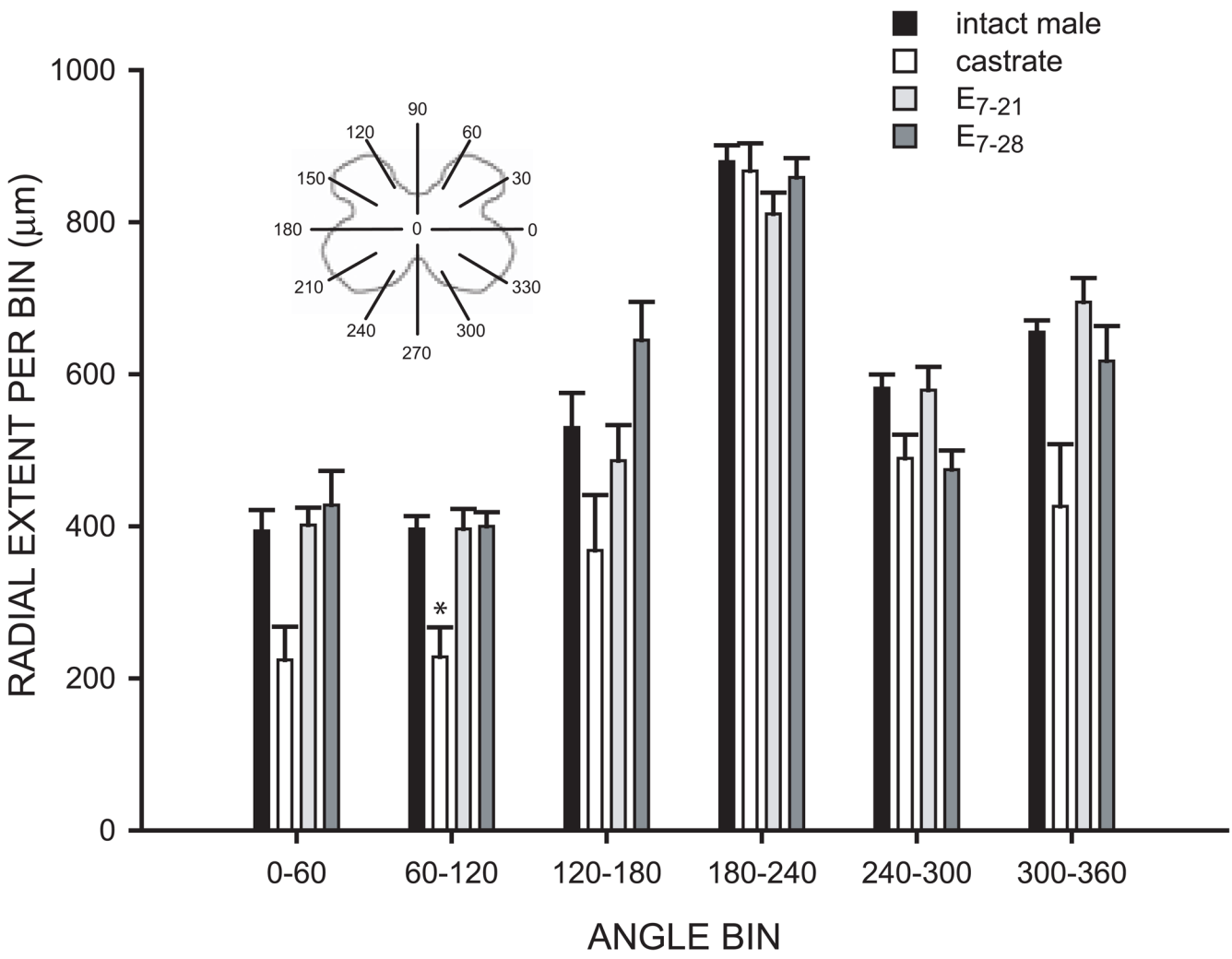

Figure 7.

(Top) Schematic drawing of spinal gray matter divided into radial sectors for measure of SNB radial extent. (Bottom) Radial extents of SNB dendrites at P28 for intact males, untreated castrates (castrate), and castrates with estradiol implants from P7-P21 ($E_{7–21}$) or from P7-P28 ($E_{7–28}$). For graphical purposes, dendritic extent measures have been collapsed into six bins of 60° each. Bar heights represent means ± SEM for four to six animals per group.