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Androgen Receptor Immunoreactivity in Skeletal Muscle: Enrichment at the Neuromuscular Junction

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ABSTRACT

Potential cellular targets of androgen action within skeletal muscle of the rat were determined by comparing the cellular distribution of androgen receptor (AR)-positive nuclei in the highly androgen-responsive levator ani (LA) muscle with that of the relatively androgen-unresponsive extensor digitorum longus (EDL) muscle. We found that androgen responsiveness correlates with AR expression in muscle fibers and not in fibroblasts. Results indicate that a much higher percentage of myonuclei in the LA are AR^+ than in the EDL (74% vs. 7%), correlating with differences in androgen responsiveness. Both muscles contain an equivalent proportion of AR^+ fibroblasts (~62%). AR^+ nuclei were not observed in terminal Schwann cells in either muscle. These results suggest that ARs within LA muscle fibers mediate the androgen-dependent survival and growth of the LA muscle and its motoneurons. We also observed an unexpected enrichment of AR^+ myonuclei and fibroblasts proximate to neuromuscular junctions, suggesting that ARs at muscle synapses may selectively regulate synapse-specific genes important for the survival and growth of motoneurons. Although castration reduced the proportion of AR^+ fibroblasts in both muscles, the proportion of AR^+ myonuclei was reduced only in the LA. As expected, testosterone treatment prevented these effects of castration but, unexpectedly, increased the proportion of AR^+ myonuclei in the EDL to above normal. These results suggest that how AR expression in skeletal muscle is influenced by androgens depends not only on the particular muscle but on the particular cell type within that muscle. J. Comp. Neurol. 473:59–72, 2004.

Indexing terms: spinal nucleus of the bulbocavernosus; sex differences; levator ani; extensor digitorum longus; synapse specificity; perineal muscle

Gonadal androgens exert a wide variety of physiological and morphological effects that culminate in the expression of reproductive behavior and function in males (Meisel and Sachs, 1994). The final common pathway controlling penile function during male copulatory behavior is the spinal nucleus of the bulbocavernosus (SNB) neuromuscular system. This system, originally described for rats (Breedlove and Arnold, 1980), is sexually dimorphic in adults and exquisitely sensitive to gonadal androgens (Christensen et al., 2000). Males have three times as many SNB motoneurons as do adult females, and SNB motoneurons in males innervate the bulbocavernosus (BC) and levator ani (LA), striated perineal muscles that surround and attach exclusively to the base of the penis. SNB motoneurons in adult females and a minority in males innervate the external anal sphincter, a muscle present in both sexes (McKenna and Nadelhaft, 1986). The BC/LA muscles not only are critical for male fertility (Sachs, 1982; Hart and Melese-D'Hospital, 1983), but also prove to be an important site where androgen acts to organize and maintain this system.

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Sex differences in the number of SNB motoneurons and target muscle fibers originate through an androgenic regulation of developmental cell death (Cihak et al., 1970; Breedlove and Arnold, 1983a,b; Nordeen et al., 1985). The SNB system develops in both sexes prenatally, but most of it dies just after birth in females. Androgen treatment of newborn females can prevent this cell death (Nordeen et al., 1985), leading to a masculinized SNB system in adult females (Breedlove and Arnold, 1983b). It is clear that the androgenic regulation of cell death requires androgen receptors (ARs), insofar as the SNB system is completely feminine in adult genetic males having a mutated AR, i.e., having the testicular feminization mutation (Tfm; Breedlove and Arnold, 1981). This mutation in the AR gene renders males insensitive to their own testicular androgens, and development of the SNB system follows a female pattern (Sengelaub et al., 1989).

Gonadal androgens acting on ARs not only orchestrate the sexually dimorphic development of the SNB system but continue to play a key role in maintaining the male phenotype in adulthood. For example, the size of SNB motoneuronal somata and the length of SNB dendrites shrink if adult males are castrated (Breedlove and Arnold, 1981; Kurz et al., 1986). The size of BC/LA muscles, their fibers and their neuromuscular junctions are also continually maintained by androgens in adulthood and will atrophy if androgens are removed (Balice-Gordon et al., 1990). Androgen replacement in adulthood will restore these morphological indices to normal (Christensen et al., 2000) as well as reinstate the expression of penile reflexes controlled by this system (Hart, 1973; Meisel et al., 1984).

Knowing where androgens act is a critical step toward identifying the molecular signals by which androgens regulate cell fate and phenotype within the SNB system. Significant progress has been made on this issue. For example, convergent pieces of evidence indicate that androgens act in development on the BC/LA muscles to ensure their survival, which secondarily ensures the survival of the SNB motoneurons (Fishman et al., 1990; Fishman and Breedlove, 1992; Freeman et al., 1996; Jordan et al., 1997). Evidence also suggests that androgens in adulthood act via ARs in BC/LA muscles to maintain both muscle fiber size and length of SNB dendrites (Rand and Breedlove, 1992, 1995). Adult SNB motoneurons also have ARs, and androgens act there in adulthood to modulate the size of their somata as well as the level of calcitonin gene-related peptide (CGRP) expression (Monks et al., 1999; Watson et al., 2001). Nonetheless, the BC/LA muscles have proved to play a decisive role in the androgenic regulation of a number of cellular events underlying the sexual differentiation and adult morphology of the SNB system.

Despite the apparent importance of the BC/LA muscles in mediating androgen action on the SNB system, little is known about the distribution and regulation of the expression of ARs in these muscles, nor do we know much about what makes the BC/LA muscles considerably more sensitive to androgens than other skeletal muscles. For example, whereas the BC/LA muscles depend on androgens in adulthood to maintain their size, most other striated muscles in rats do not (Wainman and Shipounoff, 1941). Data based on biochemical binding assays indicate that the BC/LA muscles of adult male rodents contains three to five times as many androgen-binding sites as skeletal muscles in the limb (Dube et al., 1976; Tremblay et al., 1977; Dionne et al., 1979; Rance and Max, 1984), suggesting that differences in AR complement may underlie differences in androgen responsiveness. More recent estimates of AR protein content based on Western blotting also suggest that the BC/LA is enriched for ARs compared with other skeletal muscles (Antonio et al., 1999). However, little is known about the cellular distribution of ARs in skeletal muscle and whether the cellular distribution of AR varies among muscles that differ in their androgen responsiveness. Because skeletal muscles contain several different cell types, including muscle fibers, fibroblasts, and terminal Schwann cells (TSCs), such information will help to identify the potential cellular target(s) through which androgens exert their effects on the SNB system.

Previous work in this laboratory has demonstrated robust AR immunostaining of cell nuclei in the LA muscle (Jordan et al., 1997). Although some AR^+ nuclei clearly belonged to muscle fibers, it was not clear with the approach used (thick longitudinal sections of muscle) whether other cell types in muscle also contain ARs. Moreover, whether regional differences exist in the distribution of AR^+ nuclei within muscles was not assessed. The present study compared AR immunoreactivity (-ir) in thin cross-sections of the adult LA and the extensor digitorum longus (EDL) limb muscle, two muscles that differ markedly in their androgen responsiveness. The LA is highly androgen responsive, whereas the EDL is relatively unresponsive to androgens and does not depend on androgens to maintain adult fiber size (Lubischer and Bebinger, 1999). We report that, whereas both muscles contain similar proportions of AR^+ fibroblasts, only LA muscle fibers contain substantial numbers of AR^+ nuclei. Moreover, ARs appear to be enriched at the muscle synapse, more myonuclei and fibroblasts being AR^+ near the synapse than farther away. Finally, we find that nuclear AR-ir is regulated by adult androgens in both muscles, although the pattern of change and magnitude depend on both the particular muscle and the cell type within that muscle. In general, however, AR-ir in cell nuclei of skeletal muscle requires adult circulating levels of gonadal androgens.

MATERIALS AND METHODS

Animals and tissue harvesting

At 55–60 days of age, Sprague-Dawley male rats (obtained from Charles River Laboratories) were either castrated or sham operated. Rats were anesthetized with an i.p. injection of rat ketamine cocktail (100 mg/ml ketamine, 20 mg/ml xylazine, 10 mg/ml acepromazine mg/ml; 0.09 ml/100 g body weight) and castrated through scrotal incisions. The testes were visualized but not removed for sham castrations. While still anesthetized, each animal was also given subcutaneous (s.c.) implants of two 2-cm-long (effective release length) Silastic capsules (0.062 inches inner diameter; 0.125 inches outer diameter; constructed according to Smith et al. 1978) containing either crystalline testosterone (T) or nothing (blank). Among the castrates, 10 received blank Silastic capsules and eight received capsules containing T. Nine males were sham castrated and implanted with blank Silastic capsules. Four weeks later, the animals were reanesthetized with the same ketamine cocktail, and the LA and EDL were dissected, coated in OCT embedding medium for frozen tissue specimens (Tissue-Tek), and frozen in CO_2-cooled

isopentane. Animals were overdosed with an intracardial injection of rat ketamine cocktail, and seminal vesicles were harvested and stored in 10% buffered formalin. After ≥30 days of fixation, seminal vesicles were weighed as a convenient bioassay for circulating androgen titers. All procedures used in this study have been approved by the Michigan State University All University Committee of Animal Use and Care and conform to NIH guidelines.

The muscles were held at −80°C until transversely sectioned at 8 μm with a cryostat. The sections were thaw mounted onto gel-subbed slides and stored at −80°C in slide boxes until staining. Adjacent muscle sections were distributed across a set of five slides, and five or six series of five slides were generated per muscle. LA and EDL muscles from seven or eight animals per hormone condition were sectioned and used for analysis of AR expression.

Staining skeletal muscle sections for ARs, basal lamina, acetylcholine receptors, and DNA

Because of our interest in AR expression at the neuromuscular junction (NMJ), we first screened one-fifth of the muscle sections for the presence of NMJs by using a modified cholinesterase stain (Pestronk and Drachman, 1978). On the day of staining, slide boxes containing slides of LA and EDL sections were allowed to warm to room temperature. Once warmed, LA and EDL sections were fixed for 5 minutes in 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4), rinsed twice in phosphate-buffered saline (PBS; 140 mM NaCl, 10.7 mM KCl, 1 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4), and stained for 15 minutes for cholinesterase. Staining was stopped by rinsing twice in dH_2O, and the sections were examined under a dissection scope for the presence of stained endplates. Once NMJ-containing sections were located, alternate unstained muscle sections containing endplates were quadruply labeled for the following cellular markers: AR, basal lamina (laminin B2), acetylcholine receptors (AChRs), and DNA according to procedures described below. All steps occurred at room temperature unless otherwise specified.

Sections were fixed for 5 minutes in 4% phosphate-buffered paraformaldehyde, as described above, and rinsed in PBS. We stained first for the AR using the PG21 rabbit polyclonal antiserum (generously provided by Dr. Gail Prins) directed against the first 21 amino acids of the rat and human AR as described previously (Jordan et al., 1997). PBS was used throughout for rinses and as the carrier solution for immune and nonimmune labels. Triton X-100 was omitted from the protocol, because we found that excellent staining of all cellular markers could be achieved without the aid of Triton X-100. After 48 hours of incubation in PG21 (0.8–2.2 μg/ml) at 4°C in a humidified environment, endogenous binding of biotin and avidin was blocked by using an avidin/biotin blocking kit (Vector, Burlingame, CA) at half the recommended concentration. ARs were visualized with Elite ABC peroxidase reagents (Vector), a biotinylated goat anti-rabbit IgG (1:3,200; Vector), and nickel-enhanced diaminobenzidine. After we reacted the peroxidase, tissue sections were rinsed several more times in PBS, then blocked for 30 minutes in PBS containing 0.2% bovine serum albumin (BSA) and 0.1% sodium azide (PBS/BSA solution) and incubated overnight at 4°C in the same PBS/BSA solution containing a mouse monoclonal antibody against laminin B2 [MAb D18: 1:50; Developmental Studies Hybridoma Bank, University of Iowa (Sanes et al., 1990)], and BODIPY-FL α-bungarotoxin (1:100; Molecular Probes, Eugene, OR), a fluorescein-like label used to visualize AChRs. B2 laminin was visualized by incubating tissue for 1 hour in rat-adsorbed biotinylated horse anti-mouse (1:200), followed by 1 hour of incubation in Cy3-strepavidin (1:1,800; Jackson Immunoresearch, West Grove, PA). After several more rinses in PBS, tissue was stained for 5 minutes with DAPI (1 μg/ml) to visualize cell nuclei, rinsed in dH_2O, dehydrated in graded ethanols, cleared in Citri Solv (Fisher, Fair Lawn, NJ), and coverslipped with DPX (Sigma Aldrich, St. Louis, MO). Slides were stored short term (about 1 week) at 4°C and then stored long term at –20°C to slow fading of fluorescent labels. Handled in this manner, the fluorescent tags used in this study retain a high signal to noise ratio for more than 1 year after the initial staining.

At least two slides from each muscle were stained at one time, with muscle sections from each of the three hormone conditions run at the same time. One of the two companion slides from each muscle was incubated in the vehicle solution without the AR primary antiserum (PG21). Such slides served as our "no-primary" control slides for the AR. With this one exception, all slides were subjected to all labels and all staining conditions.

Microscopic analysis

We estimated the following in cross-sections of LA and EDL muscles in which ARs, basal lamina, AChRs, and cell nuclei were stained: the percentage of AR^+ myonuclei at synaptic and nonsynaptic regions of muscle fibers and the percentage of AR^+ fibroblasts at endplate- and nonendplate-containing regions. We also estimated the diameter of LA and EDL muscle fibers. These measures are described in detail below. All analyses were carried out blind to hormone condition.

Fiber diameter was measured in cross-sections of muscle from the AR control condition (no AR staining). Sections were imaged under green fluorescent light, revealing the Cy3-labeled basal lamina of individual muscle fibers (Fig. 1A,C). Muscle fibers were sampled from four different fields of view distributed across a single muscle section. To estimate the diameter of muscle fibers, we used NIH Image to measure "ellipse minor axis" after tracing the boundary (marked by the basal lamina stain) of individual muscle fibers. We used the minor axis of the drawn ellipse as our measure of diameter, because this measure is independent of any errors that might have been introduced by the angle of sectioning. Only muscle fiber profiles without NMJs in the field of view were sampled, because fibers tend to be larger at the junction than outside the junction. Fiber profiles with NMJs were easily identified, given the marked thickening of the basal lamina at the NMJ (Fig. 1A,C) and the concentration of AChRs, revealed under blue light (Fig. 1B,D). The centralmost muscle fiber in the image was sampled first, followed by sampling of fibers contiguous with the first measured fiber, and so on, until 12 fibers were measured per image. Hence, final estimates of fiber diameter per muscle are based on a sample of 48 fibers for each muscle examined (N = 6–8 animals/group).

To estimate the percentage of AR^+ myonuclei, AR^+ and AR^- myonuclei were counted under combined transmitted and epifluorescent illumination (Fig. 2). For each section

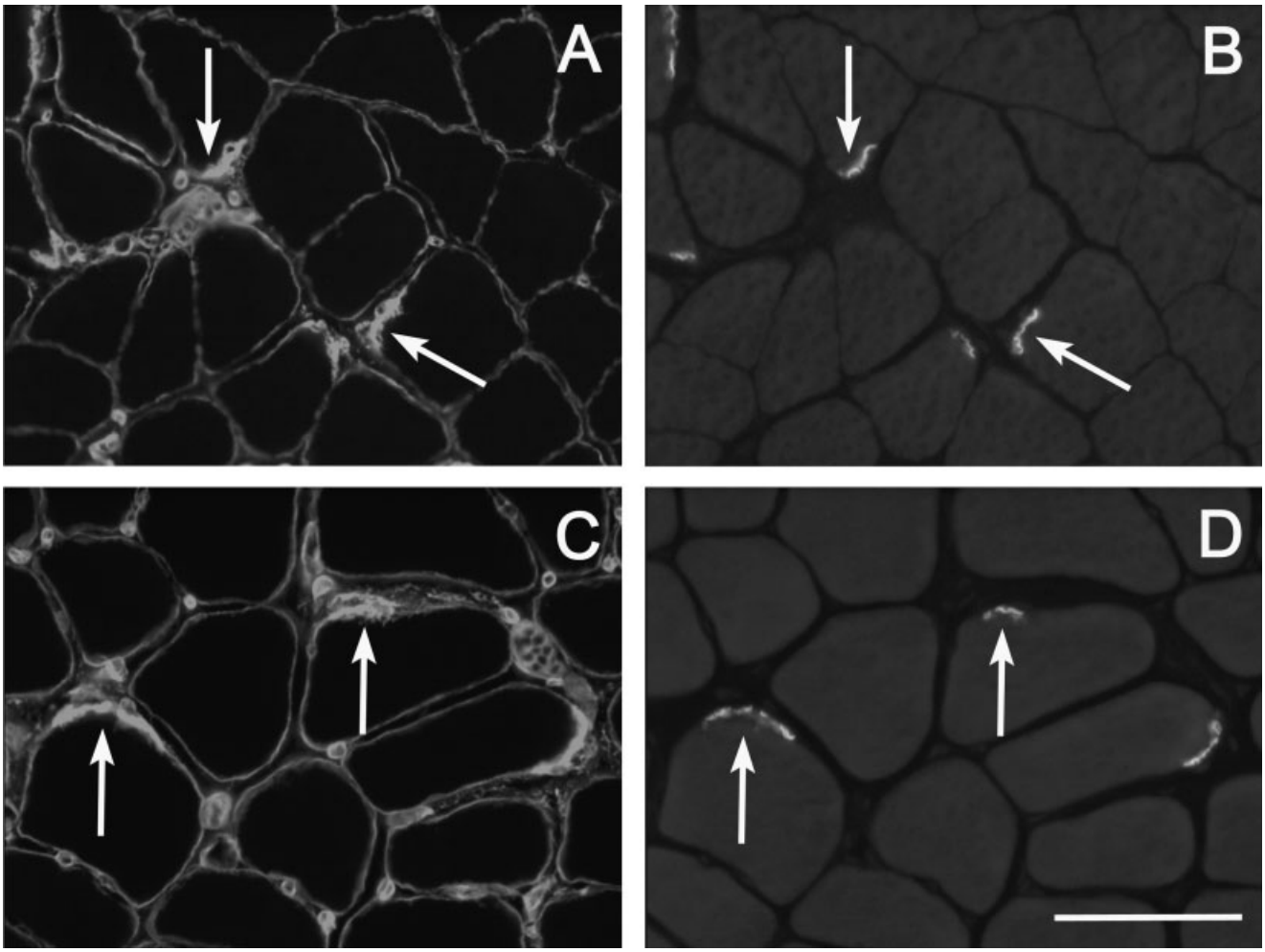

Fig. 1. Representative photomicrographs of cross-sections of the LA (**A,B**) and EDL (**C,D**) from a gonadally intact adult male rat that were costained for muscle basal lamina (A,C) and acetylcholine receptors (AChRs; B,D) and viewed under different epifluorescent illumination. Each stain clearly reveals the location of the NMJs (arrows). Note the correspondence in the location of apparent thickenings in the basal lamina of muscle fibers (arrows in A and C) with the location of AChRs (arrows in B and D). Scale bar = 100 μm.

analyzed, sections were viewed first at ×100 under brightfield illumination (no fluorescent light) to confirm uniformity of AR staining across the section. The section was then viewed at a higher magnification (×400–600) under combined illumination for AR^+ and AR^- nuclei. Muscle fibers were sampled systematically throughout the section (from left to right), and, for each fiber, myonuclei were counted as either AR^+ (presence of black reaction product within a red basal lamina ring; Fig. 2A,C,E) or AR^- (no black reaction product but DAPI stained; Fig. 2B,D,F) and classified as either synaptic or nonsynaptic. Synaptic (or soleplate) myonuclei are directly beneath the NMJ, whereas nonsynaptic myonuclei occupy other areas within the fiber (Fig. 3). One to three sections were analyzed from each muscle, with a range of 51–102 fibers sampled per muscle [mean values based on N = 6–8 muscles (animals)/group].

Nonsynaptic myonuclei were further subdivided into type I and type II nuclei (Fig. 3). Type I nuclei are nonsynaptic myonuclei within fiber profiles that have a visible junction, whereas type II nuclei are myonuclei within fiber profiles that lack a visible junction. Unless otherwise specified, reported estimates of the number of nonsynaptic AR^+ myonuclei are based on myonuclei within fiber profiles that lack a visible junction.

Our sample of myonuclei likely included nuclei of satellite cells, a class of cells with myogenic potential (Allen and Rankin, 1990). Satellite cells are located inside the basal lamina but outside the plasma membrane of muscle fibers (Ontell and Dunn, 1978). Electron microscopy is required to differentiate myonuclei from satellite cells, but they also represent a small fraction of the total population of apparent myonuclei (Nnodim, 2000).

To quantify AR staining in fibroblasts, we used the same approach, combined brightfield and epifluorescent illumination, for estimating the percentage of AR^+ fibroblasts (Fig. 2). Nuclei of fibroblasts are easily distinguished from myonuclei in that they are located outside of the basal lamina of muscle fibers, and, unlike muscle fibers, fibroblasts lack a basal lamina (Peters et al., 1991). Fibroblasts falling within a 10 × 10 ocular grid were counted as either AR^+ or AR^- in regions of the muscle

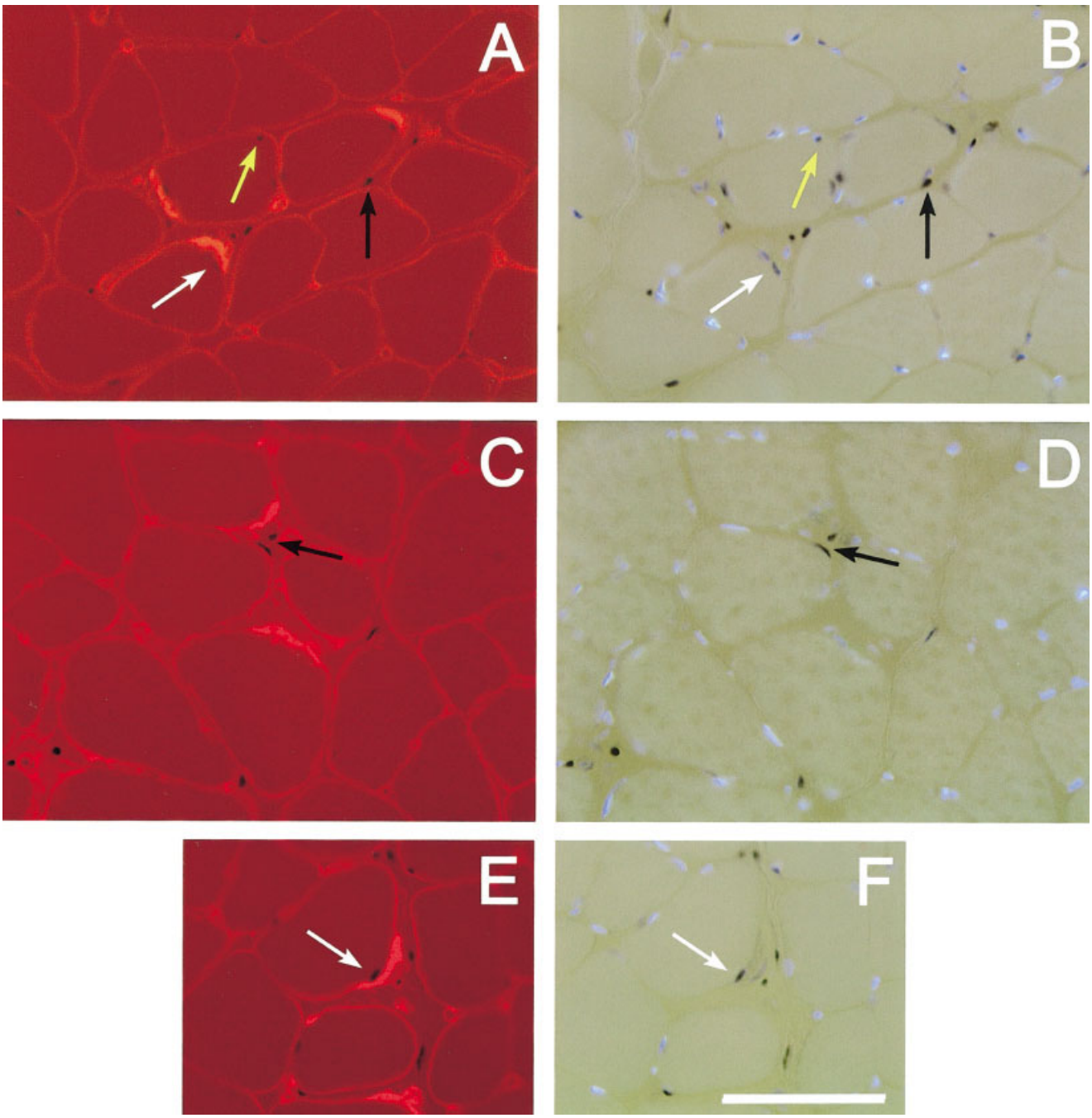

Fig. 2. Representative photomicrographs of cross-sections of the LA (**A,B**) and EDL (**C–F**) viewed under *combined* transmitted and epifluorescent illumination. Each row of photomicrographs shows the same section viewed under a different epifluorescent illumination in combination with transmitted illumination. Coillumination with epifluorescent and transmitted light allows simultaneous visualization of the black peroxidase label of ARs in cell nuclei with the Cy3 label of muscle fiber basal lamina (A,C,E) or the DAPI label of AR-negative nuclei (B,D,F), required to count AR^+ and AR^- nuclei and classify them as either myonuclei or fibroblast nuclei. Fibroblasts, unlike TSCs and pericytes, lack a basal lamina. LA and EDL cross-sections (A–D) are from a gonadally intact male and the cross-section of EDL (E,F) is from an androgen-treated castrate. Note that AR^+ nuclei in the LA (A,B) are both inside of fibers (white and yellow arrows) and outside of fibers (black arrows), indicating that both muscle fibers and fibroblasts in the LA contain AR^+ nuclei. Note also, in the LA, AR^+ synaptic myonuclei associated with the NMJ (white arrow). The normal EDL (C,D) contains prominent AR^+ nuclei in the interstitial space between muscle fibers (black arrow), suggestive of AR^+ fibroblasts, but is largely devoid of AR^+ myonuclei. In contrast, the EDL from androgen-treated castrates (E,F) contains significant numbers of AR^+ myonuclei, with marked increases in the number of AR^+ myonuclei that are both synaptic (white arrow) and nonsynaptic, reminiscent of the pattern of AR staining in the normal LA. Scale bar = 80 μm.

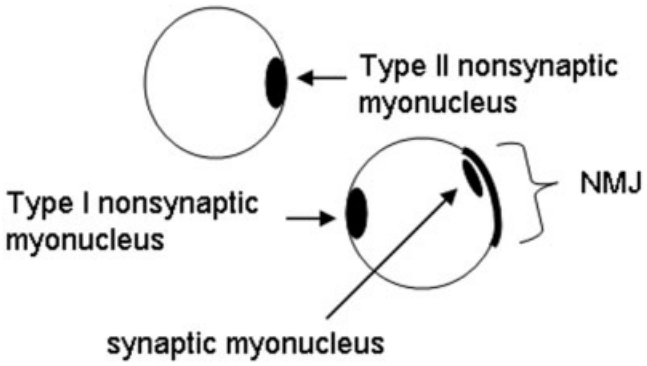

Fig. 3. Diagram illustrating our classification scheme for three different types of myonuclei in cross-sections of muscle fibers. "Synaptic" myonuclei are located directly beneath the postsynaptic junction and are transcriptionally distinct from other myonuclei outside of the postsynaptic junction (Sanes et al., 1991; Tang et al., 1994). Three to six such nuclei tend to cluster under the junction, and virtually all stain robustly for the AR in the androgen-responsive LA muscle. Nonsynaptic myonuclei are further divided into type I and type II myonuclei. Type I myonuclei are present outside of the junction but in profiles of muscle fibers that have visible NMJs. Type II myonuclei are present in muscle fiber profiles that do not have visible NMJs.

cross-section that either contained endplates or not, and 10 such fields were sampled for each region, taken from two to six sections/muscle. An endplate region was an area defined by the 10 × 10 grid that contained at least one endplate (number of fibers with endplates ranged from one to eight/sampled field); fibroblasts falling within this area are referred to as *synaptic fibroblasts*. A nonendplate region was defined by the same 10 × 10 grid and was devoid of fibers with endplates; fibroblasts falling within this area are referred to as *nonsynaptic fibroblasts*. Areas containing lengths (as opposed to cross-sections) of intramuscular nerve and blood vessels within the muscle section were excluded from our sample for both endplate and nonendplate regions of the muscle. Because only a fraction of the muscle fibers within any given cross-section have visible endplates, endplate and nonendplate regions could generally be sampled from the same sections. This analysis was carried out on a subset of the muscles (N = 3–4) used for assessing AR expression in myonuclei. Nonetheless, robust and significant differences were seen in the pattern of AR staining of fibroblasts, as reported below.

We also assessed whether TSCs stain positively for AR. TSCs, unlike fibroblasts, have a basal lamina (Peters et al., 1991) and are located directly over NMJs (Lubischer and Bebinger, 1999). We did not, however, find any evidence that TSCs stained specifically for AR, so a quantitative analysis was not carried out on this cell type.

Statistical analysis

Statistical analyses were performed using the computer program Statview (version 5). A one-way analysis of variance (ANOVA) with three conditions [sham + blank (sham/bl), castrate + blank (cast/bl), and castrate + T (cast/T)] was used to assess the effects of androgen manipulation on seminal vesicle weight and fiber size. Two-way ANOVAs with one repeated measure (endplate vs. nonendplate) were used to compare the effect of androgen manipulation on the incidence of AR^+ nuclei at and outside of the endplate. For post hoc comparisons, either paired *t*-tests or Fisher's PLSD tests were used. Separate analyses were run for each measure (percentage AR^+ myonuclei and percentage AR^+ fibroblasts) and for each muscle type (LA and EDL).

Digital photomicrography

Digital images were captured with an Optronics DEI-750 3-CCD color video camera and Scion NIH Image. Images were imported into Adobe Photoshop 5.5, where they were resized (pixels were *not* resampled), and their contrast and brightness were adjusted.

RESULTS

Cross-sections of LA and EDL muscle were stained for AR, basal lamina, AChRs, and cell nuclei. This quadruple-labeling approach allowed us to determine whether AR^+ nuclei belonged to muscle fibers, fibroblasts, and/or TSCs and to determine whether AR expression varied depending on proximity to the muscle synapse and/or androgen levels.

Examining the tissue qualitatively revealed that myonuclei in the LA are stained for AR, as previously reported (Jordan et al., 1997). Preliminary observations also suggested that a distinct population of cells outside of muscle fibers also stained for AR. Such AR^+ cells lack a basal lamina, are located in the extracellular space between muscle fibers, are associated with connective tissue, and are present in both endplate and nonendplate regions of the muscle. Thus, this population of AR^+ cells is likely to be fibroblasts (Gatchalian et al., 1989; Peters et al., 1991) and is referred to as such throughout this report. Because these cells are sometimes just outside the basal lamina of muscle fibers, whereas myonuclei are just inside, staining the basal lamina of muscle fibers was necessary to identify definitively these two classes of cell nuclei (myonuclei and fibroblast nuclei). We also noted marked AR staining of nuclei at the NMJ (Jordan et al., 1997). Based on the current approach, such AR^+ nuclei at the neuromuscular synapse are nuclei of fibroblasts and muscle fibers, located above the NMJ in cells without a basal lamina (fibroblasts) and below the NMJ in muscle fibers (myonuclei). TSCs, which also lie above the junction, *have* a basal lamina, but they did not stain for AR in either muscle. (We did observe light but nonspecific staining of TSC nuclei, as shown in Fig. 2.) AR^+ nuclei were also observed in and around blood vessels, presumably belonging to endothelial cells and pericytes. Both cell types are easily identifiable because of the presence of basement membranes, but AR expression in these cells was not quantified. Thus, detailed quantitative analysis of AR expression in the LA and EDL was limited to myonuclei and fibroblasts in and outside of synaptic regions of muscle. We also measured the size of muscle fibers as a convenient bioassay of circulating androgen titers. Values throughout are reported as mean ± SEM.

Muscle fiber diameter

The diameter of muscle fibers is a commonly used index of androgen sensitivity for skeletal muscles (Jordan et al., 1989a,b; Lubischer and Bebinger, 1999; Hegstrom et al., 2002). This measure was used to reevaluate androgen sensitivity of the LA and EDL muscles. As expected, androgen manipulations influenced the size of LA but not

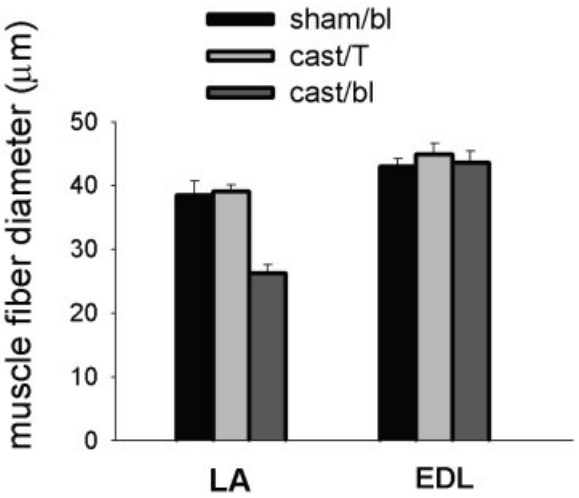

Fig. 4. Mean diameter (±SEM) of LA and EDL muscle fibers. Although the diameter of EDL fibers was not affected by changes in androgen levels, the diameter of LA fibers was. LA fibers underwent marked atrophy (30% reduction) in response to castration, whereas androgen treatment of castrates prevented this atrophy.

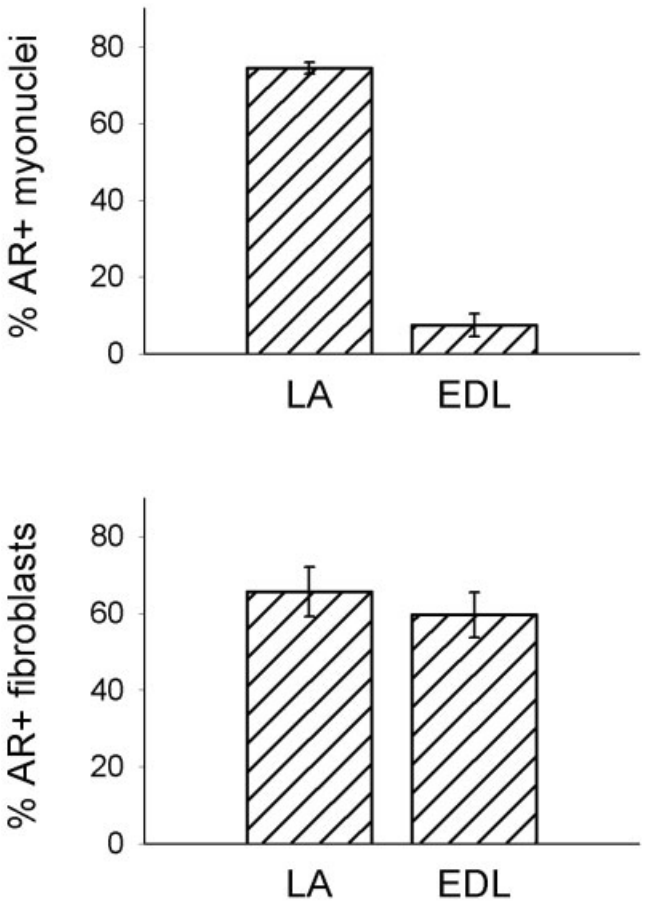

Fig. 5. Mean (±SEM) percentage of myonuclei and fibroblasts that are AR^+ in adult LA and EDL muscles from gonadally intact males. Most LA myonuclei are AR^+, whereas few are AR^+ in the EDL. In contrast, the LA and EDL both contain the same proportion of AR^+ fibroblasts. This correlation between the proportion of AR^+ myonuclei and androgen responsiveness (both are high in the LA and low in the EDL) suggests that differences in AR expression in muscle fibers may underlie differences in androgen responsiveness of these muscles and that AR in muscle fibers may mediate a number of biological responses of the SNB system to androgens, including cell survival during development and growth of motoneurons and muscles in adulthood.

EDL fibers (LA: $P < 0.001$; EDL: $P > 0.05$; Fig. 4). Four weeks after castration, the size of adult LA muscle fibers decreased (sham/bl vs. cast/bl: $P < 0.001$), whereas T treatment of castrates during the same 4-week period maintained LA fibers at a normal size (sham/bl vs. cast/T: $P > 0.05$). In sum, the size of LA fibers depends on endogenous gonadal androgens in adulthood, whereas the size of EDL fibers does not. The effects of castration and T implants on seminal vesicle weight paralleled the effect on LA fiber size (sham/blk:1.55 ± 0.08 g; cast/T: 1.89 ± 0.12 g; cast/blk: 0.15 ± 0.03 g).

AR expression in LA and EDL from sham controls

Both the LA and the EDL show distinct and apparently exclusively nuclear AR staining (Fig. 2). However, the frequency of AR^+ nuclei varies greatly between these two muscles depending on the particular cell type. Although the proportion of fibroblasts that are AR^+ is equivalent in the LA and EDL (65.55% ± 6.40% in the LA vs. 59.57% ± 5.84% in the EDL), the proportion of myonuclei that are AR^+ is much greater in the LA than in the EDL (74% ± 1.45% vs. 7% ± 3.00%, respectively; Fig. 5). These results suggest that the large majority of AR^+ nuclei in the EDL is of nonmuscle origin, if the relative frequencies of myonuclei and fibroblasts are reasonably comparable in this muscle. To investigate this possibility, we estimated the relative frequency of nuclei of muscle fibers and fibroblasts in both LA and EDL muscles from sham castrates (N = 4) and found that myonuclei outnumbered fibroblast nuclei in both muscles by less than twofold (1.95 ± 0.14 in the LA vs. 1.50 ± 0.19 in the EDL based on four sample regions per muscle). Taking these results into account, we conclude that AR^+ myonuclei predominate over AR^+ fibroblasts in the LA, whereas this pattern is reversed in the EDL.

When myonuclei and fibroblast nuclei were classified as synaptic or not, additional differences were revealed. Despite the relative paucity of AR^+ myonuclei in the EDL, both muscles show a significant difference in the percentage of AR^+ myonuclei at the endplate relative to outside the endplate region ($P < 0.001$ in the LA, $P < 0.04$ in the EDL, based on paired t-tests). In each case, more synaptic nuclei than nonsynaptic nuclei are AR^+ (Fig. 6). Moreover, a marked spatial gradient of AR staining was also evident in the two different types of nonsynaptic myonuclei (Table 1, Fig. 3). AR^+ myonuclei were most abundant at the junction, followed by outside the junction (but within fiber profiles with visible junctions) and were least in fibers without visible junctions. Given that these estimates were based on fibers sampled from the same endplate-containing muscle sections, these data suggest that the location of ARs in muscle fibers is tightly regulated. Fibroblasts also show a regional difference in AR staining, with a greater percentage of AR^+ fibroblasts in endplate-

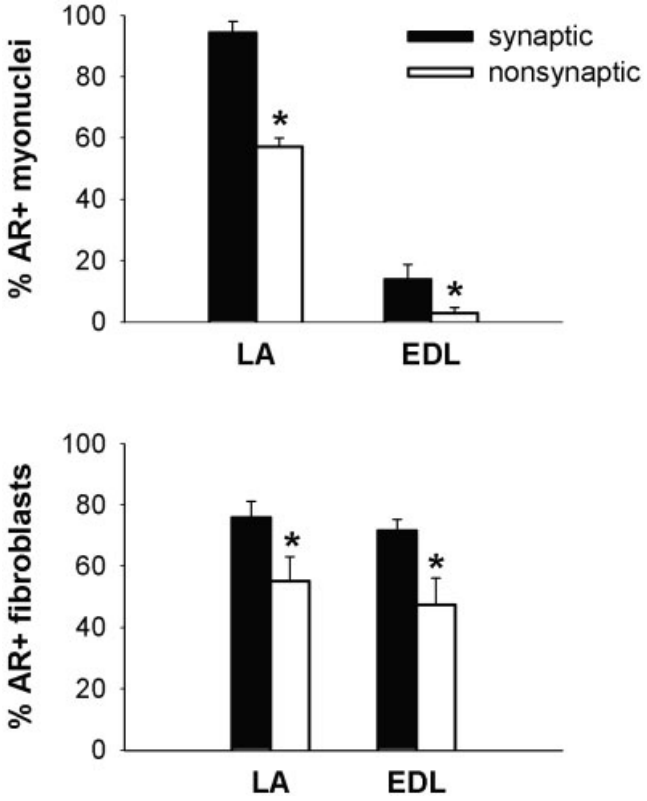

Fig. 6. Mean (±SEM) percentage of synaptic and nonsynaptic myonuclei and fibroblasts that are AR^+ in the LA and EDL from gonadally intact adult male rats. Despite the fact that few myonuclei overall are AR^+ in the EDL, the proportion of myonuclei that are AR^+ is increased in synaptic compared with nonsynaptic myonuclei in both muscles. This apparent synaptic enhancement of AR expression also extends to fibroblasts in both muscles, with a greater percentage of AR^+ fibroblasts in regions containing synapses compared with nonsynaptic regions of muscle. *Ps < 0.04, compared with synaptic.

TABLE 1. Spatial Distribution of AR^+ Myonuclei in LA and EDL Muscle Fibers and the Influence of Androgens (Mean ± SEM % AR^+ Myonuclei)

	Muscle fibers w/ visible junctions		Muscle fibers w/o visible junction (nonsynaptic; Type II)
	Synaptic	Nonsynaptic (type I)	
LA			
Sham	94 ± 3.57	72 ± 5.32	57 ± 2.89
Cast/T	93 ± 2.42	69 ± 3.41	55 ± 3.08
Cast/blk	37 ± 2.65	14.4 ± 6.99	14 ± 5.68
EDL			
Sham	12 ± 4.82	8 ± 2.87	3 ± 1.76
Cast/T	54 ± 6.00	28 ± 8.01	18 ± 6.21
Cast/blk	0.3 ± 0.33	0	0

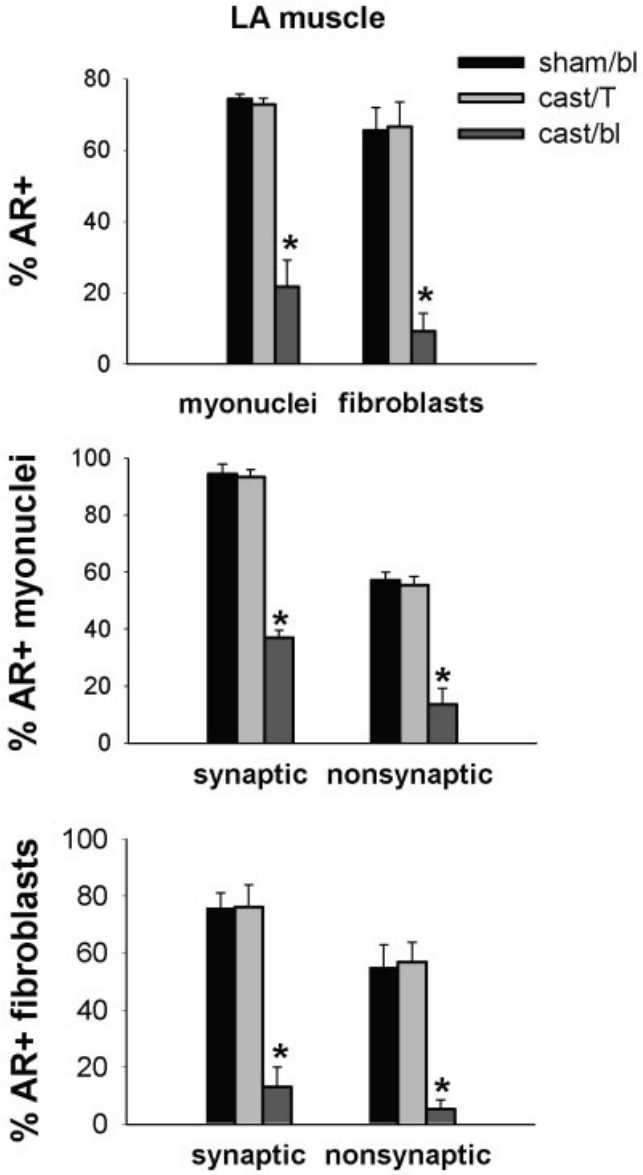

Fig. 7. Mean (±SEM) percentage of AR^+ myonuclei and fibroblasts in the LA from gonadally intact (sham/bl) males and males castrated and given either testosterone (cast/T) or blank (cast/bl) capsules for 1 month. Androgen manipulations affected similarly the proportion of AR^+ myonuclei and fibroblasts in the LA. The overall proportion of AR^+ myonuclei and fibroblasts decreased significantly after castration, whereas androgen treatment prevented this decline. The same pattern is also evident for myonuclei and fibroblasts that are synaptic or nonsynaptic. *Ps < 0.01, compared with sham/bl.

containing regions than in nonendplate-containing regions ($P < 0.015$ in the LA and $P < 0.036$ in the EDL, based on paired t-tests; Fig. 6).

Effect of androgens on AR-ir in LA and EDL

Number of AR^+ myonuclei and fibroblast nuclei in the LA muscle depends on androgens. As expected, removal of gonadal androgens had profound effects on the pattern of AR expression in the LA (main effect of treatment, $P < 0.001$; Fig. 7). After 4 weeks of castration, the proportion of AR^+ myonuclei decreased significantly in the LA (sham/bl vs. cast/bl, $P < 0.001$), whereas T treatment of castrates during this same 4-week period blocked the effect of castration, maintaining the percentage of AR^+ myonuclei comparable to that in sham males (sham/bl vs. cast/T: $P > 0.05$). AR expression in LA fibroblasts follows the same pattern as for LA myonuclei (main effect of treatment: $P < 0.001$), with the number of AR^+

fibroblasts significantly higher in sham males than in control-treated castrates (sham/bl vs. cast/bl: $P < 0.001$) but no difference between sham males and T-treated castrates (sham/bl vs. cast/T: $P > 0.05$).

For both myonuclei and fibroblasts, there were also overall main effects of region (endplate vs. nonendplate, Ps < 0.001), with no interaction between region and treatment ($P > 0.05$). Thus, similar effects of manipulating androgen on synaptic and nonsynaptic myonuclei were evident (main effect of treatment within region: Ps < 0.001; Fig. 7), with castration reducing the percentage of both (sham/bl vs. cast/bl: Ps < 0.001), and T treatment of castrates preventing the effect of castration (sham/bl vs. cast/T: Ps > 0.05). The enhanced AR staining at the NMJ was retained in LA muscles from control-treated castrates (cast/bl), despite the low number of AR^+ myonuclei overall in these LA muscles. Fibroblasts inside and outside of endplate-containing regions also showed a reduction in AR expression after castration (Ps < 0.002), with T treatment preventing this effect (Fig. 7).

Exogenous androgens increase AR staining in EDL myonuclei. Although androgen manipulations significantly influenced the number of AR^+ myonuclei in the EDL (main effect: $P < 0.001$; Fig. 8), the pattern of change was different from that seen in the LA. Androgen treatment of castrates induced a significant increase in the percentage of AR^+ myonuclei compared with sham males (sham/bl vs. cast/T: $P < 0.001$), whereas castration had no significant effect on the number of AR^+ myonuclei (sham/bl vs. cast/bl: $P > 0.05$), although this result likely reflects a floor effect, in that only about 10% of myonuclei are normally AR^+ in the EDL.

As in the LA, the proportion of AR^+ myonuclei across the three groups was significantly higher at synaptic than at nonsynaptic regions of EDL fibers (main effect: $P < 0.001$; Fig. 8). This pattern is evident in EDL muscles from both sham/bl and cast/T groups (Ps < 0.04) but not in EDL muscles from cast/bl animals, presumably because the number of AR^+ myonuclei has reached a floor at both synaptic and nonsynaptic regions of the muscle for this group (Fig. 8). Because region effects drop out in the cast/bl group, there is also a significant main interaction between treatment and region ($P < 0.001$).

Significant treatment effects are also evident for myonuclei at both synaptic and nonsynaptic regions of EDL muscle fibers (Ps < 0.01), with T-treated muscles having more AR^+ myonuclei than do either sham/bl or cast/bl muscles at either location (Ps < 0.02), whereas the proportion of AR^+ myonuclei did not differ between sham/bl and cast/bl at either location in EDL muscles (Ps > 0.05). This pattern of results is in contrast to that in the LA, where differences lie between control-treated gonadally intact and castrate males (sham/bl vs. cast/bl) and not between gonadally intact males and castrates treated with T (sham/bl vs. cast/T).

Androgens also influenced the proportion of AR^+ fibroblasts in the EDL (main effect of treatment: $P < 0.03$), but, for fibroblasts, the pattern of change is similar to that in the LA (Fig. 8). Removing endogenous androgens significantly decreased the proportion of AR^+ fibroblasts (sham/bl vs. cast/bl: $P < 0.02$), whereas androgen treatment prevented the effect of castration (sham/bl vs. cast/T: $P > 0.05$). Note, however, that, although the proportion of AR^+ fibroblasts is similar across the LA and EDL for both sham/bl and cast/T groups, the proportion of AR^+ fibroblasts in the cast/bl group remains higher in the EDL than in the LA, suggesting that EDL fibroblasts may depend less heavily on adult androgens than do LA fibroblasts for continued AR expression. Androgen status also influenced the proportion of AR^+ fibroblasts at both endplate- and nonendplate-containing regions of the muscle. EDL from

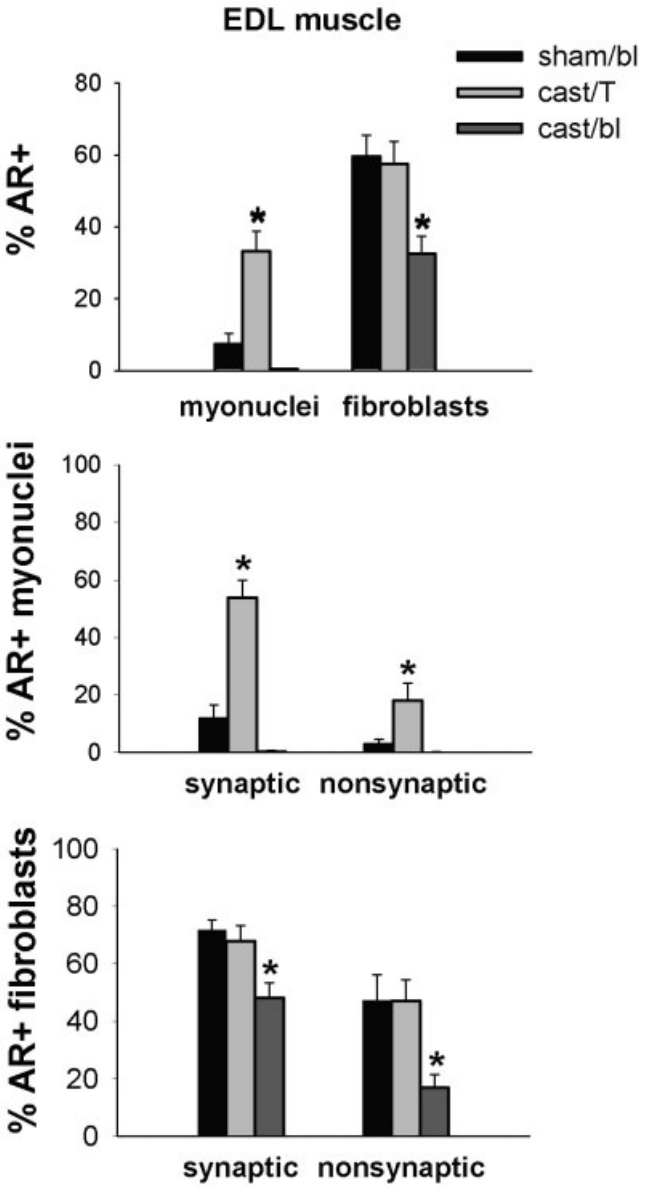

Fig. 8. Mean (±SEM) percentage of AR^+ myonuclei and fibroblasts in the EDL from gonadally intact (sham/bl) males and males castrated and given either testosterone (cast/T) or blank (cast/bl) capsules for 1 month. In contrast to the LA, the proportion of AR^+ myonuclei in the EDL was not significantly affected by castration, likely because of a floor effect, but their proportion was increased significantly by androgen treatment. Fibroblasts in the EDL followed the same pattern of change as seen in the LA, with a decreased proportion of AR^+ fibroblasts (synaptic and nonsynaptic) after castration (sham/bl vs. cast/bl group) and a normal proportion of AR^+ fibroblasts in androgen-treated castrates (sham/bl vs. cast/T). *Ps < 0.02, compared with sham/bl.

the cast/bl group have fewer AR^+ fibroblasts in both regions than do sham males (Ps < 0.001) or T-treated castrates (Ps < 0.03), with sham males and T-treated castrates having equivalent numbers of AR^+ fibroblasts in both regions of the EDL (Ps > 0.05; Fig. 8).

DISCUSSION

We used immunohistochemistry to characterize the cellular distribution of ARs in two muscles that differ in their androgen responsiveness, the LA (high androgen responsiveness) and the EDL (low androgen responsiveness). We also examined how the pattern of AR-ir is influenced by proximity to the synapse and adult androgens. We find that only the LA contains a substantial proportion of AR^+ myonuclei, although both muscles contain equivalent proportions of AR^+ fibroblasts. We also find that both muscles have a higher percentage of AR^+ nuclei in the vicinity of NMJs, involving nuclei of muscle fibers and fibroblasts. Finally, we find that removing endogenous gonadal androgens leads to a dramatic loss of nuclear AR-ir in both muscles and that, although androgen treatment of castrates reinstates the normal pattern of AR-ir in the LA, it unexpectedly induces in the EDL a pattern of AR-ir similar to that in the LA (i.e., marked AR staining of myonuclei). We did not find AR-ir in TSCs in either muscle, suggesting that this cell type in normal adult rat muscle does not express appreciable amounts of AR.

AR expression in myonuclei correlates with androgen responsiveness

The first major result is that only AR expression in myonuclei correlates with the degree of androgen responsiveness of muscles. Whereas the proportion of AR^+ fibroblasts is the same in the LA and EDL (60–65%), the proportion of AR^+ myonuclei in the LA is significantly higher than in the EDL (74% vs. 7%). We used muscle fiber diameter in this study to confirm the difference in androgen responsiveness between the LA and the EDL and find what others have previously demonstrated (Lubischer and Bebinger, 1999), that the size of adult LA fibers is maintained by circulating levels of adult gonadal androgens, whereas the size of EDL fibers is not. Androgen withdrawal for 1 month results in a 33% decrease in the diameter of LA but not EDL fibers. Previous work also indicates that androgens act directly on the muscle to regulate muscle fiber size (Rand and Breedlove, 1992). This correlation between AR expression in muscle fibers and androgen responsiveness suggests that ARs in muscle fibers may critically mediate androgen action on the SNB system. A similar approach used in frog muscles also showed that androgen-responsive muscle fibers contain more AR^+ myonuclei than unresponsive muscle fibers, similarly suggesting that androgen responsiveness of skeletal muscles may occur via muscle fiber ARs (Dorlochter et al., 1994). Future studies manipulating the expression of the AR gene in various cell types within skeletal muscle will directly test this idea.

We were unable to differentiate myonuclei from satellite cell nuclei with the present light microscopic approach (Ontell and Dunn, 1978), so some of the AR^+ myonuclei may be nuclei of satellite cells. Satellite cells play an important role in the normal growth of muscle fibers, serving as a source of nuclei that become incorporated into fibers as they grow (Moss and Leblond, 1971; Barton-Davis et al., 1999). Whereas satellite cells in vitro have been shown to express ARs (Doumit et al., 1996), they represent only a small proportion (<2%) of the total population of myonuclei in the adult rat LA (Nnodim, 2000). Nonetheless, satellite cells in the LA may express AR and mediate some of androgens' action on the SNB system.

Questions have been raised about whether the apparent anabolic effects of androgens on skeletal muscle reflect activation of the AR per se, triggering increased protein synthesis, or whether in fact androgens reduce catabolism by antagonizing the function of glucocorticoid receptors (GRs), or some combination of both (Danhaive and Rousseau, 1988; Hickson et al., 1990; Sheffield-Moore, 2000). Because proteins are continually being renewed and degraded in skeletal muscle, a shift in the balance of these two processes, either increased protein synthesis or decreased catabolism, would produce the same outcome. This controversy is still not settled, but the growth response of the BC/LA to androgens almost certainly reflects activation of AR, with consequent increases in protein synthesis (Buresova and Gutmann, 1971). The BC/LA contains specific and saturable binding sites for naturally occurring and synthetic androgens, including testosterone, dihydrotestosterone, and methyltrienolone. Such androgen binding cannot be displaced by either cortisol or the synthetic glucocorticoid dexamethasone (DEX; Dube et al., 1976; Fishman et al., 1990). Neither do androgens compete for DEX binding sites in rat skeletal muscle (Snochowski et al., 1980). Evidence based on Western blotting also confirms the presence of AR protein in rat skeletal muscle, including the BC/LA (Antonio et al., 1999). Therefore, it is likely that androgen action on the BC/LA is AR mediated, although we cannot entirely exclude the possibility that antagonism of GR function in the muscles may also contribute to the responses of the SNB system to androgens.

Synaptic enhancement of AR-ir in the LA and EDL

A second important result of this study is the apparent enrichment of ARs in cell nuclei localized at or in the vicinity of the NMJ. This regional enhancement of AR-ir involves both myonuclei and fibroblast nuclei and is evident in both muscles. The regional difference in AR^+ fibroblasts is identical in the LA and EDL muscles from gonadally intact males. About 20% more fibroblasts in the synaptic region of the muscles are AR^+ compared with fibroblasts outside of the synaptic region. AR staining is also enhanced in synaptic myonuclei, with a two- to threefold increase in the proportion of synaptic myonuclei that are AR^+ compared with nonsynaptic myonuclei. It is important to point out that these estimates (synaptic vs. nonsynaptic) were obtained from cross-sections of muscle taken within a fairly narrow region of the muscle, that containing endplates. Because each muscle section contains only a small fraction of fibers that have visible endplates, we were able to obtain such estimates of AR^+ synaptic and nonsynaptic fibroblasts and myonuclei within the same sections. In short, despite obtaining our estimates of AR^+ synaptic and nonsynaptic nuclei in muscle sections that were essentially within the endplate region of the muscle, we were nonetheless able to detect significant regionalization of AR-ir. Such differences between synaptic and nonsynaptic AR^+ nuclei might be even

greater had estimates of nonsynaptic AR^+ nuclei been taken outside of the endplate region of the muscle.

Synaptic enrichment of muscle proteins is not without precedence. A prime example is the accumulation of AChRs in the postsynaptic region of muscle fibers. Localization of AChRs at the muscle synapse is controlled largely by two signals, AGRIN and ARIA (also known as *heregulin*, a member of the neuregulin family), which are produced during development by motoneurons and released from their terminals at the developing muscle synapse (Sanes and Lichtman, 1999). Through these two signals, AChRs come to be localized strictly at the NMJ. AGRIN induces preexisting, diffusely distributed AChRs to aggregate at developing synapses (Godfrey et al., 1984; Nitkin et al., 1987), whereas ARIA up-regulates the expression of the AChR genes in synaptic myonuclei (Simon et al. 1992; Sandrock et al., 1997). At the same time, nerve-induced activity of muscle fibers down-regulates expression of the AChR genes outside of the synapse (Fromm and Burden, 1998b). Whether the apparent enrichment of ARs in synaptic myonuclei is subject to similar transcriptional regulation is currently unknown. One can imagine, however, that signals along muscle fibers might induce a certain level of AR gene expression, with additional signals from the nerve further amplifying AR gene expression postsynaptically. Candidate molecules for regulating AR gene expression in this manner are heuregulin and myogenic transcription factors, such as myogenin. Each is strongly implicated in transcriptional control of the AChR genes in synaptic and nonsynaptic myonuclei (Tang et al., 1994; Fromm and Burden, 1998a). It is clear, however, that mechanisms other than transcriptional regulation are at work in muscle to localize proteins and their transcripts to the NMJ, insofar as some are enriched at the synapse, despite transcription of the gene in both synaptic and nonsynaptic myonuclei (Barradeau et al., 2001). Hence, AR gene expression might be uniform among myonuclei with differences in recruitment and/or stability of the transcript and/or the protein causing the enhanced AR-ir postsynaptically in LA and EDL muscles.

Separate from the issue of how AR expression is locally regulated in muscle fibers is the more significant issue of what the synaptic enrichment of AR in muscle fibers might mean. Because the main difference in AR expression between the LA and EDL appears to involve myonuclei, with the LA having a higher proportion of AR^+ myonuclei than the EDL, we think it is likely that ARs in LA muscle fibers per se critically mediate androgen action on the SNB system. It is intriguing to consider the possibility that the AR, as a transcription factor, regulates the transcription of different genes in synaptic vs. nonsynaptic nuclei. In other words, ARs in adult BC/LA might have two distinct roles: ARs in synaptic myonuclei might mediate the indirect effects of androgen on adult SNB dendrites (Rand and Breedlove, 1995) by regulating the expression of synapse-specific genes for proteins that influence motoneurons, whereas ARs in nonsynaptic myonuclei might mediate the direct anabolic effects of androgens on BC/LA muscles (Rand and Breedlove, 1992) by regulating the expression of skeletal muscle genes, such as myosin, actin, and other structural proteins that are the essential building blocks contributing to fiber size and function.

Given that androgens also act via the developing BC/LA muscles to ensure the survival of the muscles and their innervating SNB motoneurons, a similar increased concentration of ARs at the synapse of developing muscle fibers would also suggest that ARs in different parts of the muscle fiber regulate different genes—muscle genes in nonsynaptic myonuclei that promote survival and growth of the muscle and other genes in synaptic myonuclei that promote the survival and growth of motoneurons. Candidate molecules that mediate the androgenic control of these cellular processes include ciliary neurotrophic factor (Forger et al., 1993) and its receptor (Forger et al., 1997; Xu and Forger, 1998), brain-derived neurotrophic factor, neurotrophin-3 (Xu et al., 2001), insulin-like growth factor-1 (IGF1; Urban et al., 1995; Arnold et al., 1996; Mauras et al., 1998; Bhasin et al., 2001; Lewis et al., 2002), and myogenin (Lee, 2002), all of which have been shown to mimic the effects of androgens on the SNB and/or the expression of which has been shown to be sensitive to androgens. The use of various promoter-AR gene constructs to control which nuclei within muscle fibers express ARs may begin to parse out which responses of the SNB system to androgens are controlled by which ARs within the muscle fiber.

Although the mechanism for how AR expression becomes enhanced in fibroblasts near the NMJ is not clear, it is clear that there are distinct populations of fibroblasts that occupy synaptic and nonsynaptic regions of skeletal muscle (Weis et al., 1991). Each population shows different patterns of gene expression and responds differently to muscle denervation (Connor and McMahan, 1987; Gatchalian et al., 1989). Factors that confer unique traits on synaptic fibroblasts may also similarly act to regulate AR expression.

The functional significance of AR expression in synaptic and nonsynaptic fibroblasts is also not clear. It is noteworthy, however, that the main population of AR^+ cells in peripheral nerve is also fibroblasts (Jordan et al., 2002b) and that androgens enhance the rate of nerve regeneration (Kujawa et al., 1993). Fibroblasts are the major source of collagen that forms the various connective sheaths present in both nerve and muscle, and androgens may regulate collagen production, which in turn influences the elasticity of peripheral nerves and skeletal muscles. Fibroblasts also produce a number of extracellular matrix molecules (ECMs), including N-CAM, tenascin, and fibronectin, that serve as adhesive molecules and are thought to have roles in the directed growth of axons (Sanes, 1989). Fibroblasts may therefore have specialized roles in directing and supporting nerve regeneration (Gatchalian et al., 1989), especially because these ECMs are laid down more heavily by synaptic fibroblasts at denervated muscle synapses, to which regenerating axons ultimately return. Androgens might therefore regulate the role of fibroblasts in this process through the transcriptional regulation of such ECM genes.

Androgens influence AR-ir in the LA and EDL

The third major finding of this study is that AR-ir in skeletal muscle cell nuclei is heavily dependent on circulating levels of adult androgen. This dependence was most obvious in the LA, with substantial decreases in the number of AR^+ myonuclei and fibroblasts after castration, with T treatment of castrates preventing these decreases.

Castration and hormone treatment also affected similarly the proportion of AR^+ fibroblasts in the EDL. Unexpectedly, androgen treatment of castrates increased significantly the proportion of AR^+ myonuclei in the EDL compared with the normal number. These results indicate that AR expression in both muscles is influenced by androgens, although the magnitude and pattern of change depend on the particular skeletal muscle under study and the particular cell type within a muscle.

If the level of AR expression in muscle fibers underlies androgen-induced growth of muscle fibers, then EDL fibers would be expected to be larger than normal after T treatment, given the marked increase in AR^+ myonuclei after T treatment in this muscle. We did not find this. EDL fibers were the same size, regardless of treatment condition. It may be that even higher levels of AR are required before EDL muscle fibers will grow in response to androgens. Whereas androgen treatment induced a significant increase in the number of AR^+ myonuclei in the EDL, the level remains well below the normal level in the LA. It is also possible, however, that AR expression in muscle fibers is not the only factor determining androgen responsiveness of these cells and that other factors, such as steroid receptor coactivators, also play a part in conferring androgen responsiveness on skeletal muscles (Heinlein and Chang, 2002).

The synaptic enhancement of AR-ir generally persisted (excepting EDL myonuclei), despite the dramatic decline in the number of AR^+ myonuclei and fibroblasts in muscles after castration. These results suggest that, in addition to androgens, some other signal, perhaps of neural origin, is capable of regulating AR expression in skeletal muscles. Considerable evidence indicates that neural and/or activity-related factors can influence AR expression in both rat and human skeletal muscles (Bernard et al., 1984; Bernard and Max, 1986; Inoue et al., 1993, 1994; Deschenes et al., 1994; Bamman et al., 2001). A skeletal muscle-specific form of IGF1 is also influenced by muscle activity, and its level tends to correlate with changes in the number and/or function of ARs (Inoue et al., 1993, 1994; Deschenes et al., 1994; Bamman et al., 2001). Levels of IGF1 are also sensitive to androgens, with androgens increasing both muscle mass and IGF1 levels (Urban et al., 1995; Arnold et al., 1996; Mauras et al., 1998; Bhasin et al., 2001). In short, there is a growing body of evidence suggesting that a skeletal muscle-specific form of IGF1 may mediate the anabolic effects of androgens and/or exercise in muscle (Barton-Davis et al., 1998; Owino et al., 2001; Goldspink, 2002; Lewis et al., 2002), and, although ARs and IGF1 both seem intimately involved in this process, the relationship between the two is not yet understood.

The effects of androgen on AR-ir in muscles are consistent with what has previously been shown using an immunoblot assay (Antonio et al., 1999). AR protein levels decrease significantly after castration in both the BC/LA muscles and the plantaris, a limb muscle, although the decline is less marked in the plantaris. Data from the present study suggest that the smaller decline in AR in the plantaris might reflect residual ARs in muscle fibroblasts. Androgen treatment, in this case with dihydrotestosterone, reversed the effect of castration but also increased the amount of AR protein to levels higher than normal in all three muscles, indicating that the number of AR^+ nuclei may not necessarily reveal changes in the level of AR protein. Whereas androgen treatment restored the number of AR^+ nuclei to normal in the LA, it may well have increased the number of ARs per nucleus to a level above normal. We did find that the darkest staining AR^+ nuclei were in androgen-treated muscles. Moreover, DHT regulated AR protein levels in a manner consistent with what we found with T replacement, suggesting that T acts as an androgen and not an estrogen to regulate AR-ir in muscle nuclei.

How androgens regulate AR expression in muscle is currently not known. Androgens could maintain and/or increase the number of AR^+ nuclei in muscle via a number of different routes, including the control of gene transcription, transcript and/or protein stability, and/or intracellular localization of AR (i.e., nuclear vs. cytosolic). Androgen binding in the cytosolic fraction has been shown to increase by about fivefold in the BC/LA and by about twofold in the EDL 30 days after adult castration (Rance and Max, 1984), suggesting that muscles from castrates may have more ARs than suggested based on immunohistochemistry and that such ARs are located in the cell cytosol. However, we did not detect increases in cytosolic staining in either muscle fibers or fibroblasts after castration, as has been reported for neurons (Wood and Newman, 1993; Freeman et al., 1995; Xiao and Jordan, 2002). Given that muscle fibers have a very high cytosol-to-nuclear ratio, the failure to detect differences in the level of cytosolic staining is not surprising, although one might have expected increases in cytosolic staining of fibroblasts after castration, given the smaller cytosol-to-nuclear ratio in these cells. The fact that we did not may indicate a net loss of ARs in these cell. Data based on Western blotting also indicate a net decline in AR protein content after castration (Antonio et al., 1999). Given that the promoter/enhancer region of the AR gene contains androgen response elements (Gobinet et al., 2002) and that androgens can also regulate the stability of both the transcript and the protein (Kemppainen et al., 1992; Prins, 2000), it is possible that all three regulatory pathways contribute to the overall pattern of AR-ir seen in muscle after castration and androgen replacement.

In summary, the present study demonstrates a correlation between AR expression in skeletal muscle fibers and androgen responsiveness. LA muscle fibers robustly express ARs and also show robust responses to androgens, including survival and growth of the LA muscle and SNB motoneurons that innervate this muscle. Because AR expression is enhanced in synaptic myonuclei, ARs there may regulate synapse-specific genes for proteins that are intimately involved in keeping SNB motoneurons alive in development and maintaining the length of their dendrites in adulthood. ARs in myonuclei outside of the synapse may regulate the expression of skeletal muscle-specific genes for structural proteins that underlie the androgenic control of muscle mass.

LITERATURE CITED

Allen RE, Rankin LL. 1990. Regulation of satellite cells during skeletal muscle growth and development. Proc Soc Exp Biol Med 194:81–86.

Antonio J, Wilson JD, George FW. 1999. Effects of castration and androgen treatment on androgen-receptor levels in rat skeletal muscles. J Appl Physiol 87:2016–2019.

Arnold AM, Peralta JM, Thonney ML. 1996. Ontogeny of growth hormone, insulin-like growth factor-I, estradiol and cortisol in the growing lamb: effect of testosterone. J Endocrinol 150:391–399.

Balice-Gordon RJ, Breedlove SM, Bernstein S, Lichtman JW. 1990. Neuromuscular junctions shrink and expand as muscle fiber size is manipulated: in vivo observations in the androgen-sensitive bulbocavernosus muscle of mice. J Neurosci 10:2660–2671.

Bamman MM, Shipp JR, Jiang J, Gower BA, Hunter GR, Goodman A, McLafferty CL Jr, Urban RJ. 2001. Mechanical load increases muscle IGF-I and androgen receptor mRNA concentrations in humans. Am J Physiol Endocrinol Metab 280:E383–E390.

Barradeau S, Imaizumi-Scherrer T, Weiss MC, Faust DM. 2001. Muscle-regulated expression and determinants for neuromuscular junctional localization of the mouse RIalpha regulatory subunit of cAMP-dependent protein kinase. Proc Natl Acad Sci U S A 98:5037–5042.

Barton-Davis ER, Shoturma DI, Musaro A, Rosenthal N, Sweeney HL. 1998. Viral mediated expression of insulin-like growth factor I blocks the aging-related loss of skeletal muscle function. Proc Natl Acad Sci USA 95:15603–15607.

Barton-Davis ER, Shoturma DI, Sweeney HL. 1999. Contribution of satellite cells to IGF-I induced hypertrophy of skeletal muscle. Acta Physiol Scand 167:301–305.

Bernard PA, Max SR. 1986. Neural control of muscle androgen receptors. J Neurochem 46:1942–1946.

Bernard PA, Rance NE, Fishman PS, Max SR. 1984. Increased cytosolic androgen receptor binding in rat striated muscle following denervation and disuse. J Neurochem 43:1479–1483.

Bhasin S, Woodhouse L, Casaburi R, Singh AB, Bhasin D, Berman N, Chen X, Yarasheski KE, Magliano L, Dzekov C, Dzekov J, Bross R, Phillips J, Sinha-Hikim I, Shen R, Storer TW. 2001. Testosterone dose–response relationships in healthy young men. Am J Physiol Endocrinol Metab 281:E1172–E1181.

Breedlove SM, Arnold AP. 1980. Hormone accumulation in a sexually dimorphic motor nucleus of the rat spinal cord. Science 210:564–566.

Breedlove SM, Arnold AP. 1981. Sexually dimorphic motor nucleus in the rat lumbar spinal cord: response to adult hormone manipulation, absence in androgen-insensitive rats. Brain Res 225:297–307.

Breedlove SM, Arnold AP. 1983a. Hormonal control of a developing neuromuscular system. I. Complete demasculinization of the male rat spinal nucleus of the bulbocavernosus using the anti-androgen flutamide. J Neurosci 3:417–423.

Breedlove SM, Arnold AP. 1983b. Hormonal control of a developing neuromuscular system. II. Sensitive periods for the androgen-induced masculinization of the rat spinal nucleus of the bulbocavernosus. J Neurosci 3:424–432.

Buresova M, Gutmann E. 1971. Effect of testosterone on protein synthesis and contractility of the levator ani muscle of the rat. J Endocrinol 50:643–651.

Christensen SE, Breedlove SM, Jordan CL. 2000. Sexual differentiationof a neuromuscular system. In: Matsumoto A, editor. Sexual differentiation of the brain. New York: CRC Press.

Cihak R, Gutmann E, Hanzlikova V. 1970. Involution and hormone-induced persistence of the M. sphincter (levator) ani in female rats. J Anat 106:93–110.

Connor EA, McMahan UJ. 1987. Cell accumulation in the junctional region of denervated muscle. J Cell Biol 104:109–120.

Danhaive PA, Rousseau GG. 1988. Evidence for sex-dependent anabolic response to androgenic steroids mediated by muscle glucocorticoid receptors in the rat. J Steroid Biochem 29:575–581.

Deschenes MR, Maresh CM, Armstrong LE, Covault J, Kraemer WJ, Crivello JF. 1994. Endurance and resistance exercise induce muscle fiber type specific responses in androgen binding capacity. J Steroid Biochem Mol Biol 50:175–179.

Dionne FT, Dube JY, Lesage RL, Tremblay RR. 1979. In vivo androgen binding in rat skeletal and perineal muscles. Acta Endocrinol 91:362–372.

Dorlochter M, Astrow SH, Herrera AA. 1994. Effects of testosterone on a sexually dimorphic frog muscle: repeated in vivo observations and androgen receptor distribution. J Neurobiol 25:897–916.

Doumit ME, Cook DR, Merkel RA. 1996. Testosterone up-regulates androgen receptors and decreases differentiation of porcine myogenic satellite cells in vitro. Endocrinology 137:1385–1394.

Dube JY, Lesage R, Tremblay RR. 1976. Androgen and estrogen binding in rat skeletal and perineal muscles. Can J Biochem 54:50–55.

Fishman RB, Breedlove SM. 1992. Local perineal implants of anti-androgen block masculinization of the spinal nucleus of the bulbocavernosus. Brain Res Dev Brain Res 70:283–286.

Fishman RB, Chism L, Firestone GL, Breedlove SM. 1990. Evidence for androgen receptors in sexually dimorphic perineal muscles of neonatal male rats. Absence of androgen accumulation by the perineal motoneurons. J Neurobiol 21:694–704.

Forger NG, Roberts SL, Wong V, Breedlove SM. 1993. Ciliary neurotrophic factor maintains motoneurons and their target muscles in developing rats. J Neurosci 13:4720–4726.

Forger NG, Howell ML, Bengston L, MacKenzie L, DeChiara TM, Yancopoulos GD. 1997. Sexual dimorphism in the spinal cord is absent in mice lacking the ciliary neurotrophic factor receptor. J Neurosci 17: 9605–9612.

Freeman LM, Padgett BA, Prins GS, Breedlove SM. 1995. Distribution of androgen receptor immunoreactivity in the spinal cord of wild-type, androgen-insensitive and gonadectomized male rats. J Neurobiol 27: 51–59.

Freeman LM, Watson NV, Breedlove SM. 1996. Androgen spares androgen-insensitive motoneurons from apoptosis in the spinal nucleus of the bulbocavernosus in rats. Horm Behav 30:424–433.

Fromm L, Burden SJ. 1998a. Synapse-specific and neuregulin-induced transcription require an ets site that binds GABPalpha/GABPbeta. Genes Dev 12:3074–3083.

Fromm L, Burden SJ. 1998b. Transcriptional pathways for synapse-specific, neuregulin-induced and electrical activity-dependent transcription. J Physiol (Paris) 92:173–176.

Gatchalian CL, Schachner M, Sanes JR. 1989. Fibroblasts that proliferate near denervated synaptic sites in skeletal muscle synthesize the adhesive molecules tenascin(J1), N-CAM, fibronectin, and a heparan sulfate proteoglycan. J Cell Biol 108:1873–1890.

Gobinet J, Poujol N, Sultan C. 2002. Molecular action of androgens. Mol Cell Endocrinol 198:15–24.

Godfrey EW, Nitkin RM, Wallace BG, Rubin LL, McMahan UJ. 1984. Components of *Torpedo* electric organ and muscle that cause aggregation of acetylcholine receptors on cultured muscle cells. J Cell Biol 99:615–627.

Goldspink G. 2002. Gene expression in skeletal muscle. Biochem Soc Trans 30:285–290.

Hart BL. 1973. Effects of testosterone propionate and dihydrotestosterone on penile morphology and sexual reflexes of spinal male rats. Horm Behav 4:239–246.

Hart BL, Melese-D'Hospital PY. 1983. Penile mechanisms and the role of the striated penile muscles in penile reflexes. Physiol Behav 31:807–813.

Hegstrom CD, Jordan CL, Breedlove SM. 2002. Photoperiod and androgens act independently to induce spinal nucleus of the bulbocavernosus neuromuscular plasticity in the Siberian hamster, Phodopus sungorus. J Neuroendocrinol 14:368–374.

Heinlein CA, Chang C. 2002. Androgen receptor (AR) coregulators: an overview. Endocrine Rev 23:175–200.

Hickson RC, Czerwinski SM, Falduto MT, Young AP. 1990. Glucocorticoid antagonism by exercise and androgenic–anabolic steroids. Med Sci Sports Exerc 22:331–340.

Inoue K, Yamasaki S, Fushiki T, Kano T, Moritani T, Itoh K, Sugimoto E. 1993. Rapid increase in the number of androgen receptors following electrical stimulation of the rat muscle. Eur J Appl Physiol Occup Physiol 66:134–140.

Inoue K, Yamasaki S, Fushiki T, Okada Y, Sugimoto E. 1994. Androgen receptor antagonist suppresses exercise-induced hypertrophy of skeletal muscle. Eur J Appl Physiol Occup Physiol 69:88–91.

Jordan CL, Letinsky MS, Arnold AP. 1989a. The role of gonadal hormones in neuromuscular synapse elimination in rats. I. Androgen delays the loss of multiple innervation in the levator ani muscle. J Neurosci 9:229–238.

Jordan CL, Letinsky MS, Arnold AP. 1989b. The role of gonadal hormones in neuromuscular synapse elimination in rats. II. Multiple innervation persists in the adult levator ani muscle after juvenile androgen treatment. J Neurosci 9:239–247.

Jordan CL, Padgett B, Hershey J, Prins G, Arnold A. 1997. Ontogeny of androgen receptor immunoreactivity in lumbar motoneurons and in the sexually dimorphic levator ani muscle of male rats. J Comp Neurol 379:88–98.

Jordan CL, Christensen SE, Handa RJ, Anderson JL, Pouliot WA, Breedlove SM. 2002a. Evidence that androgen acts through NMDA receptors to affect motoneurons in the rat spinal nucleus of the bulbocavernosus. J Neurosci 22:9567–9572.

Jordan CL, Price RH Jr, Handa RJ. 2002b. Androgen receptor messenger

RNA and protein in adult rat sciatic nerve: implications for site of androgen action. J Neurosci Res 69:509–518.

Kemppainen JA, Lane MV, Sar M, Wilson EM. 1992. Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones. J Biol Chem 267: 968–974.

Kujawa KA, Jacob JM, Jones KJ. 1993. Testosterone regulation of the regenerative properties of injured rat sciatic motor neurons. J Neurosci Res 35:268–273.

Kurz EM, Sengelaub DR, Arnold AP. 1986. Androgens regulate the dendritic length of mammalian motoneurons in adulthood. Science 232: 395–398.

Lee DK. 2002. Androgen receptor enhances myogenin expression and accelerates differentiation. Biochem Biophys Res Commun 294:408–413.

Lewis MI, Horvitz GD, Clemmons DR, Fournier M. 2002. Role of IGF-I and IGF-binding proteins within diaphragm muscle in modulating the effects of nandrolone. Am J Physiol Endocrinol Metab 282:E483–E490.

Lubischer JL, Bebinger DM. 1999. Regulation of terminal Schwann cell number at the adult neuromuscular junction. J Neurosci 19:RC46.

Mauras N, Hayes V, Welch S, Rini A, Helgeson K, Dokler M, Veldhuis JD, Urban RJ. 1998. Testosterone deficiency in young men: marked alterations in whole body protein kinetics, strength, and adiposity. J Clin Endocrinol Metab 83:1886–1892.

McKenna KE, Nadelhaft I. 1986. The organization of the pudendal nerve in the male and female rat. J Comp Neurol 248:532–549.

Meisel RL, Sachs BD. 1994. The physiolog of male sexual behavior. In: Knobil E, Neil JD, editors. The physiology of reproduction. New York: Raven.

Meisel RL, O'Hanlon JK, Sachs BD. 1984. Differential maintenance of penile responses and copulatory behavior by gonadal hormones in castrated male rats. Horm Behav 18:56–64.

Monks DA, Vanston CM, Watson NV. 1999. Direct androgenic regulation of calcitonin gene-related peptide expression in motoneurons of rats with mosaic androgen insensitivity. J Neurosci 19:5597–5601.

Moss FP, Leblond CP. 1971. Satellite cells as the source of nuclei in muscles of growing rats. Anat Rec 170:421–435.

Nitkin RM, Smith MA, Magill C, Fallon JR, Yao YM, Wallace BG, McMahan UJ. 1987. Identification of agrin, a synaptic organizing protein from Torpedo electric organ. J Cell Biol 105:2471–2478.

Nnodim JO. 2000. Satellite cell numbers in senile rat levator ani muscle. Mech Ageing Dev 112:99–111.

Nordeen EJ, Nordeen KW, Sengelaub DR, Arnold AP. 1985. Androgens prevent normally occurring cell death in a sexually dimorphic spinal nucleus. Science 229:671–673.

Ontell M, Dunn RF. 1978. Neonatal muscle growth: a quantitative study. Am J Anat 152:539–555.

Owino V, Yang SY, Goldspink G. 2001. Age-related loss of skeletal muscle function and the inability to express the autocrine form of insulin-like growth factor-1 (MGF) in response to mechanical overload. FEBS Lett 505:259–263.

Pestronk A, Drachman DB. 1978. A new stain for quantitative measurement of sprouting at neuromuscular junctions. Muscle Nerve 1:70–74.

Peters A, Palay SL, Webster HD. 1991. The fine structure of the nervous system. New York: Oxford University Press.

Prins GS. 2000. Molecular biology of the androgen receptor. Mayo Clin Proc 75(Suppl):S32–S35.

Rance NE, Max SR. 1984. Modulation of the cytosolic androgen receptor in striated muscle by sex steroids. Endocrinology 115:862–866.

Rand MN, Breedlove SM. 1992. Androgen locally regulates rat bulbocavernosus and levator ani size. J Neurobiol 23:17–30.

Rand MN, Breedlove SM. 1995. Androgen alters the dendritic arbors of SNB motoneurons by acting upon their target muscles. J Neurosci 15:4408–4416.

Sachs BD. 1982. Role of striated penile muscles in penile reflexes, copulation, and induction of pregnancy in the rat. J Reprod Fertil 66:433–443.

Sandrock AW Jr, Dryer SE, Rosen KM, Gozani SN, Kramer R, Theill LE, Fischbach GD. 1997. Maintenance of acetylcholine receptor number by neuregulins at the neuromuscular junction in vivo. Science 276:599–603.

Sanes JR. 1989. Extracellular matrix molecules that influence neural development. Annu Rev Neurosci 12:491–516.

Sanes JR, Lichtman JW. 1999. Development of the vertebrate neuromuscular junction. Annu Rev Neurosci 22:389–442.

Sanes JR, Engvall E, Butkowski R, Hunter DD. 1990. Molecular heterogeneity of basal laminae: isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. J Cell Biol 111:1685–1699.

Sanes JR, Johnson YR, Kotzbauer PT, Mudd J, Hanley T, Martinou JC, Merlie JP. 1991. Selective expression of an acetylcholine receptor-lacZ transgene in synaptic nuclei of adult muscle fibers. Development 113: 1181–1191.

Sengelaub DR, Jordan CL, Kurz EM, Arnold AP. 1989. Hormonal control of neuron number in sexually dimorphic spinal nuclei of the rat: II. Development of the spinal nucleus of the bulbocavernosus in androgen-insensitive (Tfm) rats. J Comp Neurol 280:630–636.

Sheffield-Moore M. 2000. Androgens and the control of skeletal muscle protein synthesis. Ann Med 32:181–186.

Simon AM, Hoppe P, Burden SJ. 1992. Spatial restriction of AChR gene expression to subsynaptic nuclei. Development 114:545–553.

Smith ER, Damassa DA, Davidson JM. 1978. Hormone adminstration: peripheral and intracranial implants. In: Meyer RD, editor. Methods of psychobiology. New York: Academic Press. p 259–279.

Snochowski M, Dahlberg E, Gustafsson JA. 1980. Characterization and quantification of the androgen and glucocorticoid receptors in cytosol from rat skeletal muscle. Eur J Biochem 111:603–616.

Tang J, Jo SA, Burden SJ. 1994. Separate pathways for synapse-specific and electrical activity-dependent gene expression in skeletal muscle. Development 120:1799–1804.

Tremblay RR, Dube JY, Ho-Kim MA, Lesage R. 1977. Determination of rat muscles androgen-receptor complexes with methyltrienolone. Steroids 29:185–195.

Urban RJ, Bodenburg YH, Gilkison C, Foxworth J, Coggan AR, Wolfe RR, Ferrando A. 1995. Testosterone administration to elderly men increases skeletal muscle strength and protein synthesis. Am J Physiol 269:E820–E826.

Wainman P, Shipounoff GC. 1941. The effects of castration and testosterone propionate on the striated perineal musculature in the rat. Endocrinology 29:975–978.

Watson NV, Freeman LM, Breedlove SM. 2001. Neuronal size in the spinal nucleus of the bulbocavernosus: direct modulation by androgen in rats with mosaic androgen insensitivity. J Neurosci 21:1062–1066.

Weis J, Fine SM, David C, Savarirayan S, Sanes JR. 1991. Integration site-dependent expression of a transgene reveals specialized features of cells associated with neuromuscular junctions. J Cell Biol 113:1385–1397.

Wood RI, Newman SW. 1993. Intracellular partitioning of androgen receptor immunoreactivity in the brain of the male Syrian hamster: effects of castration and steroid replacement. J Neurobiol 24:925–938.

Xiao L, Jordan CL. 2002. Sex differences, laterality, and hormonal regulation of androgen receptor immunoreactivity in rat hippocampus. Horm Behav 42:327–336.

Xu J, Forger NG. 1998. Expression and androgen regulation of the ciliary neurotrophic factor receptor (CNTFRalpha) in muscles and spinal cord. J Neurobiol 35:217–225.

Xu J, Gingras KM, Bengston L, Di Marco A, Forger NG. 2001. Blockade of endogenous neurotrophic factors prevents the androgenic rescue of rat spinal motoneurons. J Neurosci 21:4366–4372.