

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

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In the previous study (Jordan et al., 1989), we demonstrated that androgen treatment of juvenile male rats inhibits the elimination of synapses in the levator ani (LA) muscle. In the present study, we asked whether synapse elimination would occur once this juvenile androgen treatment ended. Castreated male rats were given androgen during a juvenile treatment period (7–34 d) and were killed 4 or 8 weeks after the end of androgen treatment (at 9 or 13 weeks after birth). The adult pattern of innervation in the LA was assessed (1) anatomically by counting the number of stained motor axons innervating single muscle fibers and (2) electrophysiologically by counting the number of components in intracellularly recorded endplate potentials. Based on the number of stained motor axons, the LA from juvenile androgen-treated castrates had as much multiple innervation 1 and 2 months after the end of androgen treatment (at 9 and 13 weeks) as was present during androgen treatment at 4 weeks. This suggests that no further synapse loss occurred in the LA once androgen treatment ended. Based on electrophysiological measures, adult LA muscles previously exposed to androgen were found to contain significantly more polyneuronal innervation than normal adult LA muscles. Juvenile androgen treatment also increased the size but not the number of motoneurons in the spinal nucleus of the bulbocavernosus which contains LA motoneurons. Thus, the increased level of multiple innervation in the LA is not due to a higher than normal number of motoneurons innervating this muscle. Because multiple innervation persists in the LA in the absence of continued androgen treatment, androgen may have permanently prevented synapse elimination. This observation suggests that a similar hormonal regulation of synapse elimination may contribute to the development of adult sex differences in the synaptic organization of the brain.

During development, steroid hormones are known to have permanent effects on the structural and functional organization of the adult nervous system (Arnold and Gorski, 1984; Arai et al., 1986; Arnold and Jordan, 1988; Fishman and Breedlove, 1988).

Such effects can involve changes in the number and size of adult neurons (Gurney, 1981; Jacobson et al., 1981; Breedlove and Arnold, 1983a, b; Nordeen et al., 1987), as well as in the number and specificity of synaptic connections between adult neurons and their targets (Raisman and Field, 1973; Nishizuka and Arai, 1981a, b; Breedlove, 1985; Matsumoto and Arai, 1986b). For example, gonadal androgen during a perinatal period of development irreversibly increases the size and number of adult motoneurons in the sexually dimorphic spinal nucleus of the bulbocavernosus (SNB) (Breedlove and Arnold, 1983b). Until recently, however, it has not been clear which cellular processes in development are influenced by steroid hormones. For example, hormones might alter the adult number of neurons by influencing the birth and/or survival of neurons. Recent evidence suggests that gonadal steroid hormones are capable of regulating such developmental processes as cell proliferation (Sassoon et al., 1986), neurite outgrowth (Toran-Allerand, 1976), cell death (Breedlove, 1984; Bennett and Truman, 1985; Nordeen et al., 1985) and synaptogenesis (Matsumoto and Arai, 1979; Nishizuka and Arai, 1982; DeVoogd et al., 1985; Matsumoto et al., 1988a). In this report, we evaluate the question of whether gonadal hormones might also permanently influence the adult organization of the nervous system by regulating the normal ontogenetic process of synapse elimination.

Synapse elimination occurs in many different parts of the developing nervous system, including developing neuromuscular systems (Purves and Lichtman, 1980; Thompson, 1986; Betz, 1987). During synapse elimination in muscle, a neonatal pattern of multiple innervation of single muscle fibers is converted to the adult pattern of single innervation through a selective elimination of excess synaptic connections (Redfern, 1970; Brown et al., 1976; Riley, 1977). While synapse elimination occurs in most rat muscles around the first 2 weeks after birth (Van Essen, 1982), this process occurs substantially later in the androgen-sensitive levator ani (LA) muscle of the rat (Jordan et al., 1988a). In a previous study, we provided evidence that treatment of rats with androgen during juvenile life delays synapse elimination in the LA but not in the extensor digitorum longus (EDL) (Jordan et al., 1989). In the present study, we asked whether synapse elimination would occur once the juvenile treatment ended.

In this study, we addressed this question by assessing the pattern of innervation in the LA at 1 and 2 months after the end of juvenile androgen treatment. Both histological and electrophysiological techniques were used to assess the pattern of

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innervation in the LA. We also measured the size and number of motoneurons in the SNB, which contain motoneurons that innervate the LA (Breedlove and Arnold, 1980; Schroder, 1980). We find that a high level of anatomical multiple innervation is maintained in the LA for at least 2 months after the end of juvenile androgen treatment and that such muscles show more functional polyneuronal innervation than normal.

Materials and Methods

Surgical and treatment procedures. All animals used in this study were Sprague-Dawley male rats obtained from Simonsen Laboratories or derived from pregnant dams obtained from this supplier. Procedures used for castrating and treating rats were the same as previously described (Jordan et al., 1989). In brief, rats were castrated on postnatal day (P) 7 and given daily injections of either testosterone propionate (TP) (100 μ g TP/50 gm body weight) or the vehicle oil for a period of 4 weeks, between P7 and P34. There were 2 additional control groups: normal untreated males and males that were sham castrated on P7 and given daily injections of oil during the same treatment period (P7–34). Animals were weaned at P21 and housed as litters in group cages until they were killed at 9 or 13 weeks, after an additional 4 or 8 weeks without treatment.

Histological procedures. At the time of death, animals were overdosed with sodium pentobarbital. Their LA and EDL muscles were dissected, stained with tetranitroblue tetrazolium (TNBT), and prepared for microscopy as previously described (Jordan et al., 1988a). The unperfused lumbar/sacral portion of the spinal cord was also removed from some of these animals and was put in a 0.135 M sodium phosphate-buffered solution (pH 7.2) containing 10% formalin. After several weeks of formalin fixation, spinal cords were frozen and sectioned transversely at 50 μ m thickness using a sliding microtome. Alternate spinal cord sections were mounted on coated slides and stained with either thionin or cresyl violet.

Microscopic analysis. LA and EDL muscles and spinal cords obtained from 4–6 animals from at least 2 different litters per group were analyzed without knowledge of treatment conditions. The procedure used for analyzing TNBT-stained muscles was the same as previously described (Jordan et al., 1989) and involved measuring for each sampled muscle fiber the number of different axons and preterminal branches from the same axon that contacted the fiber, the number of junctional sites formed by such axons on a fiber, and, using a calibrated ocular micrometer, the distance between junctional sites on a fiber and the width of muscle fibers. Some of the data obtained from normal males has previously been reported in a related study (Jordan et al., 1988a).

The size of SNB motoneurons is permanently increased by androgen secretions just before and after birth, and is also regulated by androgen in adulthood (Breedlove and Arnold, 1981, 1983b; Kurz et al., 1986; Forger and Breedlove, 1987; Lee et al., 1988). For this reason, the spinal cords from juvenile androgen- or oil-treated castrates at 9 weeks were compared with another control group of 9-week-old males castrated at P35 (the time at which treatment ended for males castrated on P7). Thus, all 3 groups were without gonadal androgen during the 4 weeks prior to death. Such late-castrated males were left untreated during the juvenile treatment period (P7–34). Comparable procedures were used for sacrificing, removing, and processing the spinal cords of late-castrated males. Based on alternate cross sections of the lumbar spinal cord, the number of large, densely staining neurons that had distinct nuclei were counted in the region of the SNB. Such motoneurons are located in the dorsomedial aspect of the ventral horn in lumbar segments 5 and 6 (Breedlove and Arnold, 1981). Counts of SNB motoneurons were corrected according to Konigsmark (1970) as previously described (Breedlove and Arnold, 1981). Between 25 and 30 SNB cells were also chosen at random throughout the rostrocaudal extent of the nucleus for measuring the cross-sectional area of their somata and nuclei using a digitizing tablet interfaced with a computer.

Data obtained from muscles were statistically analyzed using an analysis of variance (ANOVA) design (Keppel, 1982) and as previously described (Jordan et al., 1989). Two-way ANOVAs (treatment \times age) were carried out unless otherwise specified. Data on the number and size of SNB motoneurons were statistically analyzed using independent 2-tailed *t* tests.

Electrophysiological procedures. Multiple innervation was also assessed electrophysiologically in LA muscles from juvenile androgen-

treated and normal adult males which did not contribute to the histological data. For these experiments, animals were castrated and treated daily with TP during the 4 week juvenile treatment period (P7–P34) or were left untreated and served as normal control males. Experiments were performed when animals were 9–12 weeks old, following 4–7 weeks without treatment. The LA was rapidly dissected from deeply anesthetized (50 mg sodium pentobarbital/kg body weight, i.p.) but respiring animals. At the end of the dissection, animals were given a lethal dose of anesthetic. Dissected LA muscles were pinned as flat whole-mounts in Sylgard-coated dishes that contained oxygenated (95% O₂/5% CO₂) PBS (Card, 1977). During pinning and all subsequent stages of the experiment, LA muscles were constantly perfused with fresh oxygenated PBS. For recording, muscle contractions were blocked by exposure to PBS containing *d*-tubocurarine ($6\text{--}7 \times 10^{-7}$ M). These concentrations were near threshold for blocking muscle contractions and yielded the largest subthreshold endplate potentials. The number of synaptic inputs to a fiber was determined using standard intracellular recording procedures (Redfern, 1970; Brown et al., 1976; Balice-Gordon and Thompson, 1988), which involved counting the number of discrete reproducible changes in the amplitude of endplate potentials produced by systematically varying the strength and polarity of nerve stimulation. Intracellular recording of resting and endplate potentials were made using 0.6 M K₂SO₄-filled glass microelectrodes of 25–50 M Ω resistance, and the whole LA nerve was stimulated (100 μ sec square pulses) using a suction electrode. Hoffman modulation-contrast optics (400 \times) were used to visualize the region of nerve terminals and to facilitate microelectrode placement adjacent to the junctional site. Superficial muscle fibers were sampled across the entire surface of the LA from 5 normal and 4 juvenile androgen-treated castrated males, with 15–65 fibers sampled per muscle (mean, 36 fibers). Muscle fiber resting potentials were generally between –70 and –80 mV. Those fibers with resting potentials more depolarized than –60 mV were not used. A 1-way ANOVA was used to determine significant differences.

Results

Pattern of innervation in TNBT-stained LA and EDL muscles

Number of axons innervating LA and EDL muscle fibers. Our objective was to determine whether the level of multiple innervation changed after the end of androgen treatment. To accomplish this, we compared the level of multiple innervation in LA muscles 4 and 8 weeks after the end of treatment (at 9 and 13 weeks) with that in the LA at P28, during androgen treatment (data reported in Jordan et al., 1989). In adult (9 and 13 week) LA muscle, multiply innervated fibers were those fibers contacted by 2 or more different myelinated axons (Fig. 1). Axons were considered to be different if they were not seen to come from the same parent axon.

In LA muscles from juvenile androgen-treated (JAT) castrates, the incidence of multiply innervated fibers did not change significantly between 4 (P28) and 13 weeks (Fig. 2, $p > 0.05$), indicating that the high level of multiple innervation in androgen-treated LA muscles at 4 weeks was maintained for at least 2 months beyond the end of treatment. At both 9 and 13 weeks, androgen-treated LA muscles had a significantly higher percentage of multiply innervated fibers than normal LA muscles ($p < 0.001$ for comparisons between JAT and normal muscles at 9 and 13 weeks), a difference similar to that previously reported for androgen-treated and normal LA muscles at 4 weeks (Jordan et al., 1989). Moreover, a substantial proportion of the multiple innervation in adult JAT muscles (13–17% of total muscle fibers) involved 3 or more axons, whereas the multiple innervation in normal adult LA muscles involved only 2 axons.

In contrast to the stable high level of multiple innervation in JAT LA muscles, the level of multiple innervation in the LA from normal males and oil-treated castrates declined between 4 and 13 weeks (Fig. 2). For normal LA muscles, the percentage of multiply innervated fibers remained stable between 4 and 9

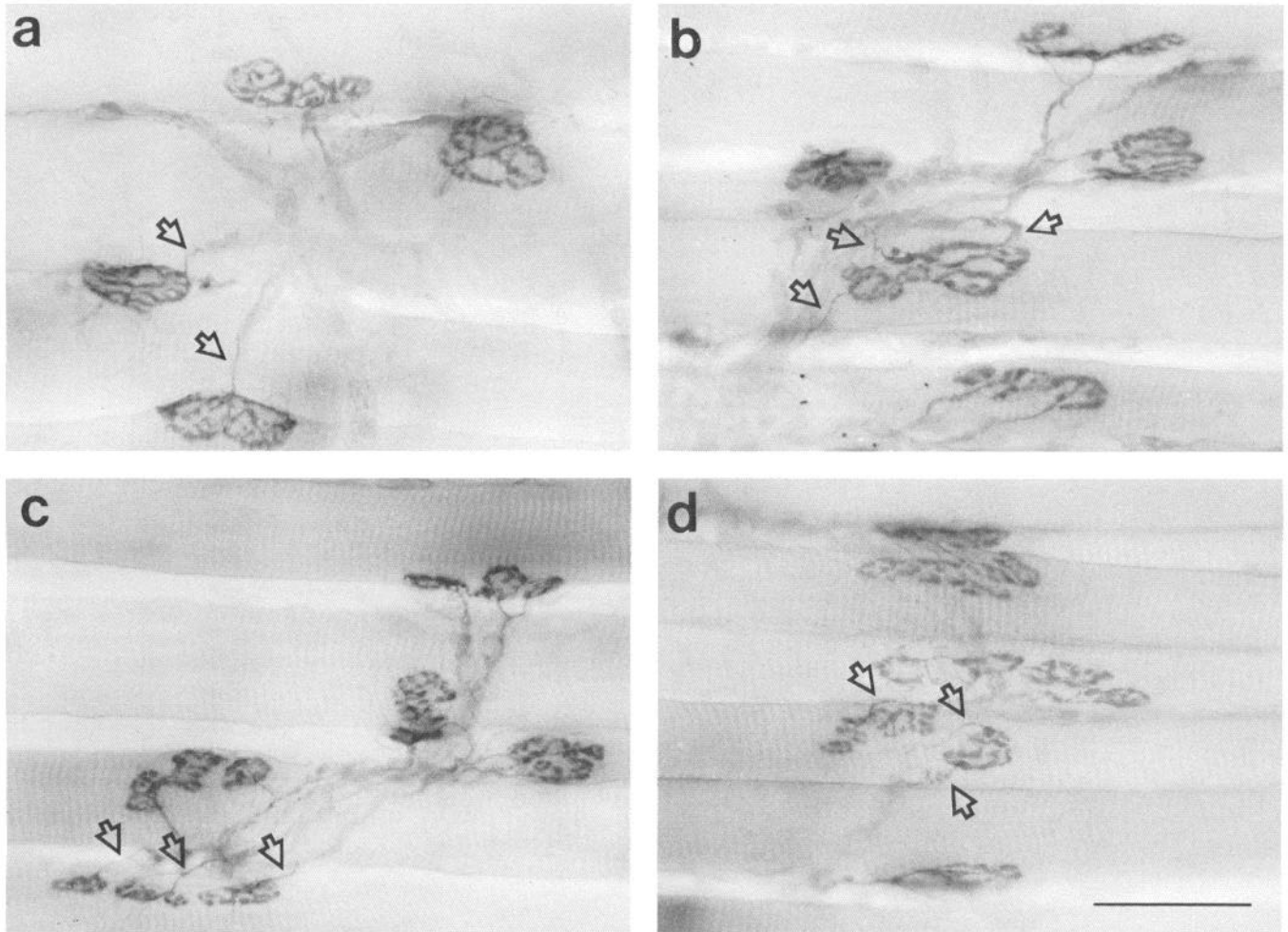


Figure 1. TNBT-stained LA motor nerve terminals in 9-week-old normal males (*a*) and adult males previously treated with androgen (*b–d*). *a*, Single motor axons (arrows) contact individual LA muscle fibers to arborize at single synaptic or junctional sites, representing examples of singly innervated fibers. *b*, Three different myelinated motor axons (arrows) contact an individual LA muscle fiber to arborize at a single junctional site. Note that the morphology of the terminal arborization formed by 3 axons (*b*) is similar to the morphology of the terminal arborizations formed by single axons (*a*). *c* and *d*, Examples of multiple innervation that involve multiple junctional sites. In *c*, 3 axons (arrows) contact an individual LA muscle fiber at 3 separate sites, to form distinctly separate, but closely spaced, terminal arborizations. Each site is singly innervated. In *d*, 3 axons (arrows) innervate an individual LA muscle fiber, but in this case, innervation is at only 2 junctional sites. This results in single innervation of one site and multiple innervation of the other. Such multiple innervation that involves 3 or more axons was present only in adult LA muscles previously treated with androgen and not in normal adult LA muscles. Calibration bar, 50 μ m.

weeks ($p > 0.05$) but dropped significantly between 9 and 13 weeks ($p < 0.009$). The LA from juvenile oil-treated castrates experienced a similar loss, although it occurred earlier, between 4 and 9 weeks ($p = 0.08$; Fig. 2). This resulted in LA muscles from oil-treated castrates having significantly less multiple innervation than normal at 9 weeks ($p < 0.005$). In contrast, at 9 weeks the LA muscles from gonadally intact, juvenile oil-treated controls contained the same amount of multiple innervation as normal ($p > 0.05$; Fig. 3), indicating that the treatment procedures themselves did not contribute significantly to the observed changes in LA innervation. Juvenile treatment did not significantly alter the pattern of innervation in EDL muscles at 9 weeks [normals: $3 \pm 1.4\%$ (mean percent multiple innervation \pm SEM); oil-treated controls: $0.2 \pm 0.2\%$; androgen-treated castrates: $1 \pm 1.4\%$; oil-treated castrates: $2 \pm 0.8\%$, $p > 0.05$, 1-way ANOVA]. The percentage of multiply innervated muscle fibers was not measured for EDL muscles at 13 weeks.

Number of junctional sites on a fiber. Androgen treatment

during synapse elimination induced the formation of closely spaced multiple junctions on some multiply innervated LA fibers (Jordan et al., 1989). Such junctions were also found to persist beyond the end of androgen treatment. At 9 and 13 weeks, LA muscles from JAT castrates contained multiply innervated fibers that possessed either single or multiple junctional sites (Fig. 1), in approximately the same proportions as observed in androgen-treated muscles at 4 weeks (Fig. 3; Jordan et al., 1989). As for multiple junctional sites in androgen-treated muscles at 4 weeks, such multiple junctions in JAT adult muscles were generally less than 10 μ m apart and were considerably more closely spaced than the multiple junctions occurring on LA fibers in normal adult muscles (which were 45 μ m apart on average). Furthermore, the multisite innervation in JAT adult LA muscles showed the same sort of heterogeneity as seen in androgen-treated muscles at 4 weeks: Multiple junctions were formed either by different myelinated axons or by unmyelinated preterminal branches from the same axon (Fig. 1), and about a

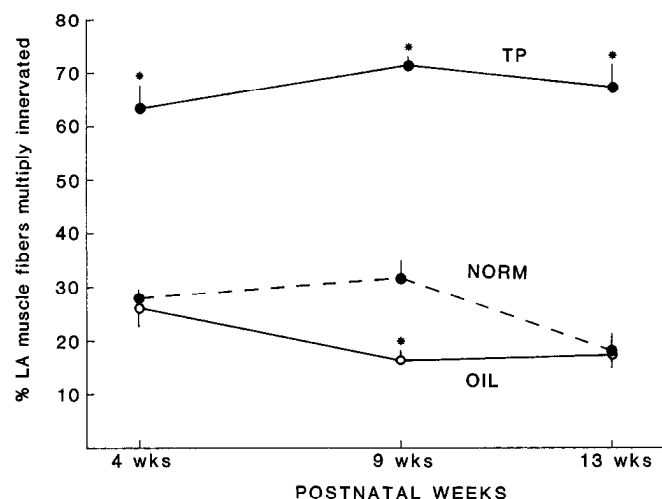


Figure 2. Percentage of multiple innervated fibers in TNBT-stained LA muscles 4, 9, and 13 weeks after birth, derived from counts of the number of motor axons synapsing on individual muscle fibers. Each data point represents the mean (\pm SEM) of muscles from 4–7 animals per group. The data at 9 and 13 weeks were obtained 4 and 8 weeks after the end of androgen (or oil) treatment, whereas the data at 4 weeks (from Jordan et al., 1989) was obtained during treatment. Multiple innervation persists after the end of juvenile androgen treatment (TP), whereas the secondary phase of synapse loss occurs a month earlier in muscles that lack endogenous gonadal androgen (OIL) than in untreated, normal (NORM) muscles. Asterisks indicate significant differences from normal.

third of the multisite fibers received convergent innervation at one or more sites on the same fiber. Figure 3 presents the percentage of multiple innervated fibers in LA muscles that have either single or multiple junctional sites.

At 9 and 13 weeks, the LA from JAT castrates had significantly more multiple innervated fibers of both types than normal muscles at these ages ($p < 0.001$ for single junctional site fibers, $p < 0.001$ for multiple junctional site fibers at both adult ages), and this was comparable to the quantitative differences observed at 4 weeks (Fig. 3; Jordan et al., 1989). For JAT LA muscles, the proportions of multiply innervated fibers having either single or multiple junctional sites did not change significantly with age (4–13 weeks, $p > 0.05$; Fig. 3).

Number of preterminal branches innervating LA fibers. The increased level of multiple innervation in androgen-treated LA muscles at P28 was also correlated with an increased percentage of LA fibers innervated by axons that branched preterminally (Jordan et al., 1989). Such preterminal branching in adult LA muscle occurs distal to the last segment of myelin and gives rise to multiple unmyelinated branches that innervate the same fiber (Jordan et al., 1988a). Preterminal branching therefore involved short ($<10 \mu\text{m}$) unmyelinated branches from the same parent axon, whereas multiple innervation involved 2 or more myelinated axons (length $\geq 20 \mu\text{m}$), possibly from different parent axons. At 9 and 13 weeks, the incidence of LA fibers innervated by preterminal branches in JAT muscles was significantly higher than in normal muscles of the same age ($p < 0.05$ at 9 weeks, $p < 0.001$ at 13 weeks; Fig. 4), indicating that preterminal branching remained at an enhanced level, despite the lack of androgen. The incidence of preterminal branching in LA muscles from other groups (juvenile oil-treated castrates or sham castrates) was similar to that found in normal LA muscles of

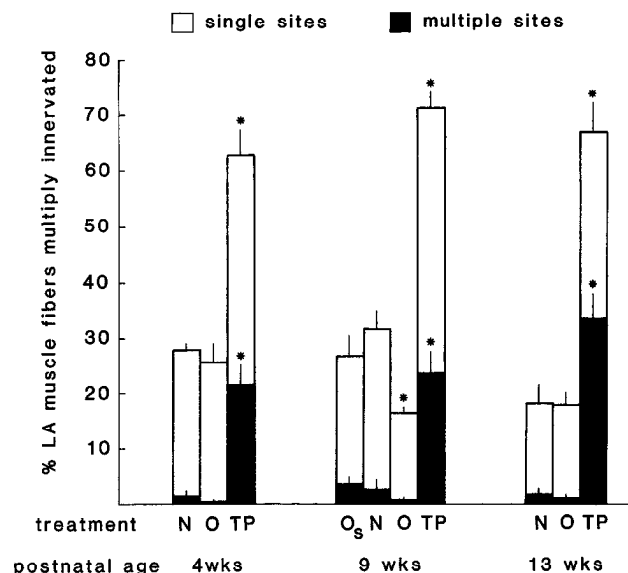


Figure 3. Percentage of multiple innervated LA muscle fibers having single or multiple junctional sites. N indicates muscles from normal males; O_s, muscles from oil-treated sham castrates; O, muscles from oil-treated castrates; and TP, muscles from androgen-treated castrates. The height of the bar indicates the total mean percentage of multiply innervated fibers. The open part of the bar indicates that portion of multiply innervated fibers having single junctional sites (\pm SEM), and the filled part of the bar indicates that portion of multiply innervated fibers having multiple junctional sites (\pm SEM). Both types of multiple innervation persist in the LA after the end of juvenile androgen treatment.

the same age ($p > 0.05$ for all comparisons at 9 and 13 weeks). Interestingly, only androgen-treated muscles exhibited an overall increase in the level of preterminal branching between 4 and 13 weeks ($p < 0.02$).

Electrophysiological data

Although extensive electrophysiological recording from these junctions has not been done, preliminary experiments confirm the effect of androgen in prolonging the period of multiple (polyneuronal) innervation. Adult LA muscles previously treated with androgen contained significantly more polyneuronal innervated fibers than normal adult muscles [$18 \pm 1.1\%$ (mean \pm SEM) and $2 \pm 1.4\%$, respectively; $p < 0.001$]. Figure 5 shows a typical example of a multicomponent EPP indicating that a fiber was polyneuronal innervated. No more than 2 synaptic inputs per fiber were ever detected electrophysiologically.

Pattern of LA muscle fiber growth

Between 4 and 13 weeks, the pattern of muscle fiber growth depended on whether gonadal androgen was present beyond the end of puberty (after 9 weeks). Although LA fibers in all groups increased in diameter between 4 and 9 weeks ($p < 0.001$ for each group), only LA fibers in normal muscles (and not castrates) continued to increase in diameter between 9 and 13 weeks ($p < 0.001$, Fig. 6). This result suggests that there are 2 distinct periods of growth for LA muscle fibers, one between 4–9 weeks in which growth of LA fibers can occur independent of androgen and the other between 9–13 weeks in which fiber growth requires androgen.

The effect of JAT on the diameter of LA muscle fibers persisted beyond the end of treatment, since at 9 and 13 weeks,

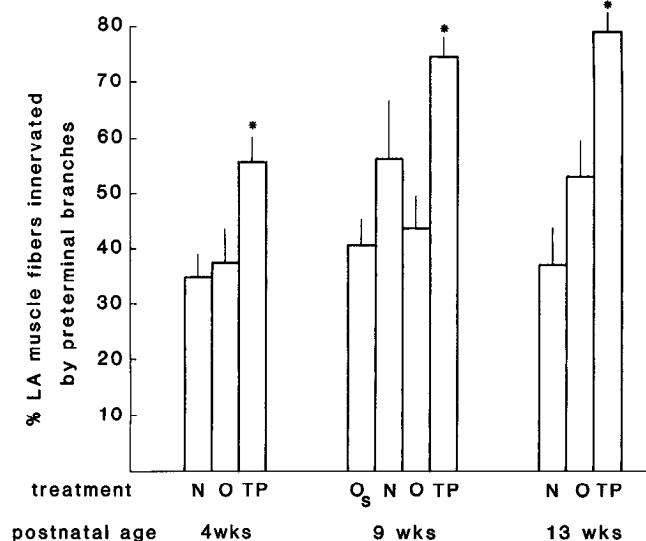


Figure 4. Percentage of LA muscle fibers innervated by unmyelinated preterminal branches from the same axon. Symbols for treatment groups are the same as indicated for Figure 3. The height of the bar represents the mean percentage of fibers innervated by preterminal branches (\pm SEM) based on muscles from 4–7 animals per group. Asterisks indicate significant differences from normal. The increased level of preterminal branching in androgen-treated muscles at 4 weeks (during androgen treatment) remains significantly higher than normal at 9 and 13 weeks, which is after the end of treatment.

LA fibers in JAT muscles were significantly larger than LA fibers in muscles from oil-treated castrates ($p < 0.001$; Fig. 6). However, at these adult ages, LA fibers in JAT muscles were significantly smaller than normal ($p < 0.03$ and 0.001 , respectively; Fig. 6). Their smaller than normal size probably reflects the lack of adult gonadal androgens, since castration in adulthood reduces the size of LA fibers (Wainman and Shipounoff, 1941; Venable, 1966; Galavazi and Szirman, 1971). Juvenile treatment did not influence the size of EDL fibers at 9 weeks [normals: $41 \pm 0.7 \mu\text{m}$ (mean \pm SEM); oil-treated sham castrates: $42 \pm 0.8 \mu\text{m}$; androgen-treated castrates: $44 \pm 1.8 \mu\text{m}$; oil-treated castrates: $39 \pm 0.7 \mu\text{m}$; $p > 0.05$, 1-way ANOVA].

SNB motoneuron number and size

The number of SNB motoneurons at 9 weeks was the same in all groups, despite differences in hormone treatment during the juvenile period (P7–34) (Fig. 7). Juvenile androgen-treated castrates had the same number of SNB motoneurons as juvenile oil-treated castrates or as late-castrated controls ($p > 0.05$ for both comparisons), suggesting that the increased level of multiple innervation in JAT LA muscles is probably not due to an increased number of motoneurons. This finding is consistent with previous work showing that the adult number of SNB motoneurons in female spinal cord is increased by androgen only during a perinatal period that ends before P7 (Breedlove and Arnold, 1983b). However, the size of SNB somata at 9 weeks did depend on the juvenile treatment (Fig. 7). The cross-sectional areas of SNB somata in JAT castrates were significantly larger than in juvenile oil-treated castrates ($p < 0.02$). They were also larger than in late-castrated controls, although not significantly ($p > 0.05$). The cross-sectional areas of nuclei of SNB motoneurons at 9 weeks were not significantly influenced by juvenile androgen [$180 \pm 17.6 \mu\text{m}^2$ (mean \pm SEM) for an-

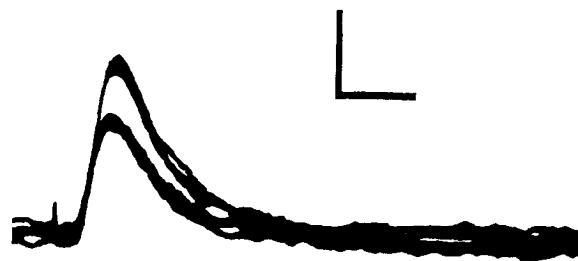


Figure 5. Multiple superimposed oscilloscope traces of intracellularly recorded EPPs from a single LA muscle fiber of a 10-week-old muscle previously exposed to androgen treatment. As stimulus strength was increased, the amplitude of the EPP also increased in a stepwise fashion, indicating that this particular LA fiber was innervated by at least 2 different motor axons. Such polyneuronally innervated fibers were more frequent in adult LA muscles previously treated with androgen than in normal adult muscles. Vertical calibration bar, 2 mV; horizontal calibration bar, 5 msec.

drogen-treated castrates compared with $145 \pm 5.0 \mu\text{m}^2$ for oil-treated castrates ($p > 0.05$) or compared with $175 \pm 17.6 \mu\text{m}^2$ for late-castrated controls ($p > 0.05$).

Discussion

The present experiments show that the adult pattern of innervation in the LA, but not in the EDL, is influenced by early androgen exposure. Much of the multiple innervation present in the LA at 2 weeks of age is maintained by androgen treatment during the period of synapse elimination (P14–28) (Jordan et al., 1989) and is retained in these muscles for at least 2 months beyond the end of treatment. This result suggests that androgen permanently prevented synapse elimination in the LA.

Alterations in the adult pattern of innervation in the LA

Counting the number of separate TNBT-stained axons contacting individual fibers in LA muscles from 9- and 13-week-old males revealed that while LA muscles from normal and oil-treated castrates continue to experience some degree of synapse loss beyond P28, muscles from JAT castrates do not experience any further loss of synapses. The percentage of multiply innervated LA fibers does not change significantly in androgen-treated muscles between 4 and 13 weeks, despite the fact that muscles after 5 weeks are no longer exposed to gonadal androgen (Fig. 2). Since the percentage of multiply innervated fibers is a measure that would not reveal losses in the number of higher-order (3 or more) axonal inputs, we also calculated the mean number of axons innervating LA muscle fibers (a measure that is sensitive to overall changes in axon number) in androgen-treated muscles at 4, 9, and 13 weeks. This number also did not change appreciably after the end of androgen treatment [1.72 ± 0.06 (\pm SEM), 1.85 ± 0.04 , and 1.87 ± 0.05 , respectively], strongly suggesting that juvenile androgen treatment has rendered the innervation pattern stable and no longer susceptible to further loss.

The processes underlying synapse elimination in the LA are apparently normally active beyond P28, since there is a continued although modest decline in the level of multiple innervation in the LA of normal males between 9–13 weeks (Fig. 2). This secondary phase of synapse loss occurs a month earlier in muscles from oil-treated castrates, between 4–9 weeks (Fig. 2), who have been deprived of the pubertal surge of androgen that occurs between 4 and 9 weeks after birth (Corpechot et al., 1981).

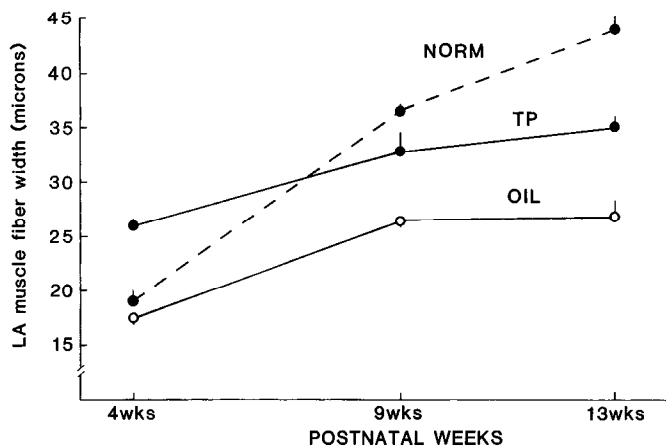


Figure 6. Pattern of LA muscle fiber growth based on measurements of the diameter of individual fibers. Each data point indicates the mean (\pm SEM) fiber diameter based on muscles from 4–7 animals per group. Labels for treatment groups are defined as in Figure 2. There are 2 distinct periods of LA muscle fiber growth, one between 4–9 weeks in which fiber growth can occur independent of androgen, and the other between 9–13 weeks in which growth requires androgen. The effect of juvenile androgen on LA muscle fiber size persists beyond the end of treatment, since TP muscles at 9 and 13 weeks contain larger muscle fibers than OIL muscles.

Therefore, this pubertal increase in androgen may normally delay the secondary period of synapse loss. Earlier phases of synapse elimination also appear to begin sooner than normal in LA muscles from oil-treated castrates (Jordan et al., 1989), suggesting that endogenous androgen both before and during puberty play a role in determining the normal time course of this process.

Previous studies have suggested that physical separation of convergent inputs to a cell is a means for mitigating competitive processes that would otherwise lead to the removal of such convergent innervation (Brown et al., 1976; Kuffler et al., 1977; Grinnell et al., 1979; Hume and Purves, 1981). For example, multiple innervation by 2 foreign nerves is preserved in muscle if the innervation occurs at separate sites along the fiber (Kuffler et al., 1977). Similarly, separation of convergent inputs appears to be an important factor ensuring the preservation of multiple innervation in autonomic ganglia (Hume and Purves, 1981), in which different axons innervate single neurons only if they can occupy different regions of the dendritic arbor (Purves and Hume, 1981; Hume and Purves, 1983; Forehand and Purves, 1984). Thus, one might have expected that multiple innervation at single junctional sites would be preferentially eliminated once hormone treatment ended, whereas multiple innervation at separate junctional sites might be more likely to be maintained after treatment. This expectation was not supported by the data. Multiple innervation of both forms are maintained in the LA after the end of androgen treatment (Fig. 3). Moreover, the degree of separation (often only a few microns) between convergent inputs on LA muscle fibers is much less than the amount of separation thought to be required ($>1000 \mu\text{m}$) to maintain multiple innervation in muscle (Brown et al., 1976; Kuffler et al., 1977). Although separation of convergent inputs may well be an important mechanism for ensuring the long-term maintenance and stability of multiple innervation in some systems, it is clearly not a requirement in the LA. Indeed, conclusions

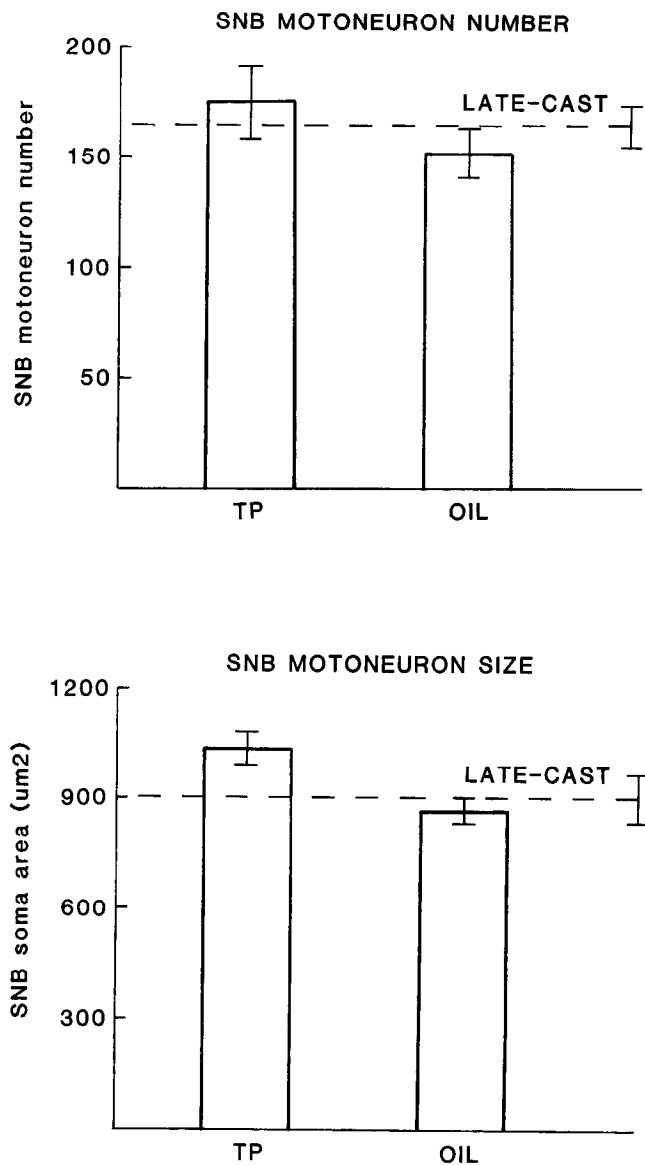


Figure 7. Number and size of SNB motoneurons in lumbar spinal cord of 9-week-old males. The height of the bars indicates the mean number or somatic area of SNB motoneurons in males castrated at P7 and treated with androgen (TP) or oil (OIL) between P7 and 34. The dashed line indicates the mean number or somatic area of SNB motoneurons for males that were untreated during the juvenile period (P7–34) and were castrated at P35 (LATE-CAST). Mean estimates (\pm SEM) based on spinal cords from 4–5 animals per group. At 9 weeks, TP castrates had larger but no more SNB motoneurons than oil-treated castrates.

similar to our own have been made about the spacing of persistent multiple inputs in adult frog sartorius muscle (Werle and Herrera, 1987).

Preliminary electrophysiological recordings revealed significantly more polyneuronal innervation in JAT adult LA muscles (18%) than in normal adult LA muscles (2%). This finding presumably reflects the fact that androgen prevented the normal loss of polyneuronal innervation during synapse elimination. Although our physiological data corroborates our anatomical data in showing the predicted difference in polyneuronal or multiple innervation, they suggest levels of polyneuronal innervation considerably lower than our anatomical estimates.

One possible explanation for this discrepancy is that some of the multiple innervation that we observe anatomically actually represents innervation by branches from the same motor axons. However, it seems more likely that the electrophysiological method used (whole nerve stimulation and curare to block muscle twitch) yielded substantial underestimates in the degree of polyneuronal innervation (Herrera, 1984). Polyneuronal inputs to mature muscle fibers tend to produce smaller endplate potentials than the inputs to singly innervated fibers (Taxt et al., 1983; Weakly and Yao, 1983; Trussell and Grinnell, 1985). These small amplitude inputs may have been difficult to detect in the present experiments.

Neuromuscular inactivity produces a similar delay in synapse elimination, but unlike androgen treatment, has not been found to prevent this process irreversibly. Thompson (1985) reported that the loss of multiple innervation in the rat soleus muscle was virtually completely blocked during a 9 day period of inactivity, but once the activity block was removed, synapse elimination occurred outside of the normal period of synapse elimination. This finding suggests that the effects of androgen and inactivity on synapse elimination may be fundamentally different. However, it is important to note that the duration (4 weeks) of androgen treatment in the present experiment was much longer than the duration of the activity block in Thompson's (1985) study. Thus, a longer period of inactivity may have a similar irreversible effect on synapse elimination. Conversely, shorter periods of androgen treatment might only transiently delay synapse elimination, but this seems unlikely. Androgen treatment lasting for only 2 weeks (P7–20) is also effective in producing high levels of multiple innervation in the LA at 9 weeks (Jordan et al., 1988b).

There are several possible mechanisms to explain androgen's long-lasting effect on synapse elimination. Since neuromuscular inactivity prevents synapse elimination (Thompson, 1985), one possibility is that androgen has permanently silenced motor nerve or muscle activity. Although we have not tested this idea directly, this possibility seems unlikely. The LA is part of the SNB neuromuscular system, which mediates penile flip reflexes that are normally active during male copulatory behavior (Sachs, 1982; Hart and Melese-d'Hospital, 1983). Both the penile reflexes and copulation depend on androgen for their activation (Beach and Levinson, 1950; Hart and Haugen, 1968; Hart, 1978; Sachs, 1978). Moreover, the physiological effects of androgen on the LA muscle and its nerve terminals argue that androgen's role is to activate rather than silence the SNB system (Vyskocil and Gutmann, 1977; Souccar et al., 1982a, b). If androgen influences synapse elimination in the LA through an effect on neuromuscular activity, it may be more likely that androgen acts to synchronize rather than block the activity of SNB motoneurons and/or LA muscle fibers. Findings from a number of studies suggest that synchronous activation may be a mechanism for maintaining convergent innervation (Hubel and Wiesel, 1965; Hubel et al., 1977; Stryker and Strickland, 1984). In this regard, it is noteworthy that adult androgen treatment increases the number of morphologically identified gap junctions found between SNB motoneurons (Matsumoto et al., 1988b). It is also possible, however, that androgen influences synapse elimination through a mechanism unrelated to neuromuscular activity, possibly by permanently increasing the amount of muscle-supplied trophic substance for which motor terminals are presumed to compete during synapse elimination (Jansen et al., 1978; Purves, 1986).

Size of SNB motoneurons

Juvenile androgen treatment produced a small but significant increase in the size, but not number, of SNB motoneurons (Fig. 7). Because of the correlation between the size of a motoneuron and the size of its motor unit (McPhedran et al., 1965; Wuerker et al., 1965; Yao and Weakly, 1986), juvenile androgen treatment probably also increased the size of motor units, which would be consistent with the high number of multiply innervated fibers in JAT LA muscles. However, the effect of androgen on SNB soma size need not correlate with changes in motor unit size or its effects on synapse elimination. For example, normal males and JAT castrates at 9 weeks have LA muscles with very different amounts of multiple innervation (Fig. 2) and, therefore, motor units that probably differ greatly in size. Both, however, have SNB motoneurons with the same mean soma area [$1026 \pm 36 \mu\text{m}^2$ ($\pm\text{SEM}$) vs. $1042 \pm 43 \mu\text{m}^2$, respectively]. Moreover, androgen can increase the size of SNB motoneurons outside of the period of synapse elimination (Breedlove and Arnold, 1981; Kurz et al., 1986) without influencing the pattern of innervation in the LA (C. L. Jordan, unpublished observations). Although these findings argue against the possibility that androgen's effect on synapse elimination is through its effect on SNB soma size, they do not exclude the possibility that the androgen-induced increase in SNB soma size that occurs *during* synapse elimination may have a role in determining the number of motor terminals a motoneuron can maintain. Since SNB motoneurons and the LA muscle have androgen receptors in adulthood (Dionne et al., 1979; Breedlove and Arnold, 1980), each represents a potential site for androgen's effect on synapse elimination and the size of SNB motoneurons.

A possible mechanism for sexual differentiation of the brain

Over the last 3 decades it has become evident that sex differences in behavior result from structural sex differences in the nervous system (Arnold and Gorski, 1984). One of the earliest reports of a sex difference in brain organization involved differences in the number of synapses (Raisman and Field, 1973). In the preoptic area of the hypothalamus in rats, adult females have more nonstriatal inputs onto dendritic spines than do males. Moreover, this sex difference is reversible by manipulating the hormonal environment during the first weeks following birth, but not in adulthood. Other regions of the brain show sex differences in the opposite direction. For example, males have more synapses than females in the ventrolateral–ventromedial nucleus of the hypothalamus and the medial amygdaloid nucleus, and such differences are also determined by gonadal hormones during postnatal development (Nishizuka and Arai, 1981a, b; Matsumoto and Arai, 1981, 1986a, b).

Adult sex differences in the synaptic connectivity of the brain might be produced through a hormonal control of such processes as synaptogenesis, cell death, and/or synapse elimination. Indeed, evidence suggests that both cell death and synaptogenesis can be regulated by gonadal hormones (Matsumoto and Arai, 1979; Nishizuka and Arai, 1982; Breedlove, 1984; DeVogd et al., 1985; Nordeen et al., 1985; Matsumoto et al., 1988a). The results of the present experiment suggest that gonadal hormones may also alter the adult number of synapses through a regulation of synapse elimination. Since synapse elimination also occurs in the brain (e.g., Crepel et al., 1976; Jackson and Parks, 1982), our results raise the possibility that synapse elimination in other steroid-sensitive parts of the nervous system may be subject to

a similar regulation by hormones, thereby establishing adult sex differences in the synaptic organization of the brain.

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