Testosterone Regulates Terminal Schwann Cell Number and Junctional Size during Developmental Synapse Elimination

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Key Words
Neuromuscular junctions \cdot Androgens \cdot Immunostaining \cdot Synapse stabilization \cdot Sexual dimorphism

Abstract
Previous work has shown that exposure to exogenous testosterone during synapse elimination permanently stabilizes synapses that would normally be lost in the androgen-sensitive levator ani (LA) muscle, indicating that testosterone is a potent stabilizing factor for developing LA synapses. Terminal Schwann cells (TSCs), which cap the neuromuscular junction, have also been implicated in the control of synaptic stability and may play a decisive role in the selective stabilization of synapses during synapse elimination. In this study, we begin to investigate the possible role of TSCs in the effect of testosterone on synapse elimination by determining whether testosterone influences their number. As the number of TSCs generally correlates with the size of endplates, we also measured endplate size. Male rats were castrated or sham gonadectomized at postnatal day (P) 7 and given capsules containing either testosterone or nothing. Three weeks later (P27–28), LA neuromuscular junctions (NMJs) were stained using immune and non-immune markers. As expected, testosterone treatment during synapse elimination maintained synapses that would ordinarily be eliminated. In addition, we find that the size of LA endplates and the number of TSCs per LA junction were also increased by the testosterone treatment. However, testosterone significantly increased the number of TSCs on both singly and multiply innervated fibers, indicating that the effect of testosterone on the number of TSCs is not specific to its effect on synapse maintenance. Rather the testosterone-induced increase in the number of TSCs appears related to the size of LA NMJs, a relationship that has precedence.

Introduction
The neuromuscular junction consists of three cellular elements: muscle, neuron and terminal Schwann cell (TSC; also known as perisynaptic Schwann cells). Despite precise registration of these three elements, our perspective of synaptic function has historically focused on the contributions made by muscle and neuron, while ignoring the potential contributions of TSCs. Only recently have we begun to appreciate that the state of the neuromuscular synapse at any one moment reflects a dynamic interaction among all three cellular elements, and that TSCs, and synaptic glia in general, may even be a decisive element in...
the formation and maintenance of synapses [Ullian et al., 2001; Trachtenberg and Thompson, 1997].

A growing body of evidence suggests that synaptic glia, which include both TSCs that cover peripheral synapses and astrocytes that cover central synapses, are intimately involved in virtually every facet of synapses, from their genesis and differentiation to their long-term maintenance and function [Ullian et al., 2001; Koenig et al., 1998; Robitaille, 1998; Chapron et al., 1997; Trachtenberg and Thompson, 1997]. For example, recent evidence indicates that TSCs in mature muscle respond to denervation by extending processes which provide a substrate that promotes axonal reinnervation of denervated endplates [Reynolds and Wolf, 1992; Son and Thompson, 1995a, b; O’Malley et al., 1999; Love and Thompson, 1999; Koirala et al.; 2000]. In contrast, TSCs in early postnatal muscle die in response to nerve injury [Trachtenberg and Thompson, 1996], which may account for the poor regenerative capacity of young axons to reform functional connections with their targets [Dennis and Harris, 1980]. On the other hand, treatment with exogenous neuregulin prevents the denervation-induced death of TSCs [Trachtenberg and Thompson, 1996].

Several pieces of evidence suggest that TSCs may have a role in synapse maintenance during developmental synapse elimination, when synapses are selectively eliminated or maintained. In contrast to the effect of neuregulin on TSCs in denervated muscles, neuregulin treatment of innervated muscles during synapse elimination induces TSCs to migrate away from their junctions and nerve terminals to withdraw [Trachtenberg and Thompson, 1997]. Similarly, transplanted Schwann cells (SCs) mimic the effect of exogenous neuregulin, inducing resident SCs and nerve terminals to move or regress from the junction. Importantly, such effects are seen only in juvenile muscle during the period of synapse elimination, and not at adult junctions [Son and Thompson, 1995b; Trachtenberg and Thompson, 1997]. Finally, in mice deficient in ErbB2, a neuregulin receptor expressed by SCs, motor synapses form prenatally in the absence of SCs but are not maintained [Lin et al., 2000]. These results suggest that SCs may be intimately involved in maintaining synapses during synapse elimination.

Synapse elimination at the neuromuscular junction (NMJ) involves a loss of polyneuronal innervation. While adult mammalian muscle fibers are typically innervated by only one motoneuron, they are polyneuronally or multiply innervated at birth [Redfern, 1970]. During the first weeks following birth, motoneurons withdraw a large portion of their axonal arbor until each muscle fiber is singly innervated, as in adulthood. We have found that synapse elimination in the rat levator ani (LA) muscle is profoundly sensitive to steroidal androgens. If endogenous androgen titers are lowered by removing the gonads from pre-pubertal male rats early in the synapse elimination process, then synapse elimination begins and ends sooner in the LA muscle [Jordan et al., 1989a, b]. On the other hand, raising androgen titers to approximately adult levels during the synapse elimination period prevents much of this process: nearly 70% of LA fibers remain multiply innervated and such multiple innervation persists indefinitely without further exposure to testosterone [Jordan et al., 1989b; Lubischer et al., 1992]. Hence, testosterone exerts a potent stabilizing influence on developing synapses during synapse elimination in the LA.

In this study, we begin to explore the potential role of TSCs in the effect of testosterone on synapse elimination by determining whether testosterone influences TSC number. Recent evidence indicates that the number of TSCs increases at the NMJ during synapse elimination [Hirata et al., 1997; Love and Thompson, 1998]. The size of junctions also increases during synapse elimination, suggesting that as a synapse expands, more terminal Schwann cells are needed to adequately ensheathe and maintain that synapse, despite the steady decline in the number of axons that innervate individual muscle fibers. Although junctional size appears to be the predominant factor regulating the number of TSCs throughout the life span [Love and Thompson, 1998; Lubischer and Bebin-ger, 1999], additional, and as yet unidentified factors also appear to contribute to the regulation of TSC number [Love and Thompson, 1998].

In this study, we asked whether altering the course of synapse elimination in muscle also alters the developmental accumulation of TSCs. In particular, when synapse elimination has been prevented by testosterone in the LA muscle, is the number of TSCs influenced and what is the relationship between the size of endplates, the number of TSCs and the number of axonal inputs? Present results replicate the androgenic sparing of LA synapses during synapse elimination. Furthermore, we find that testosterone treatment during synapse elimination increases the number of TSCs per LA junction and the size of LA junctions. However, testosterone treatment significantly increases the number of TSCs and the size of junctions on both singly and multiply innervated LA muscle fibers (i.e., on both singly innervated fibers where synapses were not spared by testosterone and multiply innervated fibers where synapses were spared by testosterone). We conclude that while testosterone increases the number
of TSCs during synapse elimination, this increase is apparently unrelated to the stabilization of LA synapses by testosterone.

**Methods**

**Animals and Hormone Treatment**

On postnatal day 7 (P7, day of birth designated as P1), male rat pups born in our colony from Sprague-Dawley dams purchased from Simonsen Laboratories (Gilroy, Calif., USA) were anesthetized with Metofane and castrated or sham gonadectomized. While anesthetized, animals received Silastic capsules (10 mm total length, 5 mm effective release length, outer diameter = 0.077 inches; inner diameter = 0.058 inches), containing either testosterone (Sigma) or nothing. There were 3 treatment conditions and the males from 5 litters were evenly distributed across the 3 groups: (1) sham castrated plus blank capsule; (2) castrated plus blank capsule and (3) castrated plus testosterone capsule. Approximately 3 weeks later, on P27 or P28, juvenile rats were deeply anesthetized with an intraperitoneal injection (0.9 ml/kg body weight) of ketamine cocktail (100 mg/ml ketamine, 20 mg/ml xylazine, 10 mg/ml acepromazine). Thereafter, two androgen-sensitive targets, the seminal vesicles and the LA muscle, and a muscle relatively insensitive to androgens, the extensor digitorum longus (EDL) muscle [Jordan et al., 1989a, b] were harvested for analysis.

Seminal vesicles were transfected to 10% phosphate-buffered formalin and allowed to fix for at least 1 month prior to being weighed. Dissected muscles were transferred to a Sylgard-coated petri dish containing cold oxygenated mammalian Ringer's solution and pinioned flat. Muscles were fixed as whole mounts for 30 min at room temperature with 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4) and stored overnight at 4°C in 0.1 M phosphate buffer containing 20% sucrose. The LA and EDL muscles were sectioned longitudinally (40 and 60 μm thick, respectively) using a freezing sliding microtome and their NMJs were visualized using immune and nonimmune probes.

**Immunocytochemistry**

The NMJs of LA and EDL muscles were visualized using a modified protocol [Lubischer and Bebinger, 1999]. Sections from each muscle were divided into three series and stained to visualize the following components of the NMJ: (1) SCs, axons and nerve terminals (fig. 1A, B), (2) SCs and endplates as marked by the presence of acetylcholine receptors (AChRs, fig. 1C, D) or (3) just axons and nerve terminals. NMJs in the first two series were visualized using fluorescent tags (fig. 1) and cell nuclei were stained using 4,6-diamidino-2-phenylindole (DAPI). Separate series were necessary due to constraints on the number of fluorescent tags easily visualized. The third series was visualized using horseradish peroxidase (HRP) histochemistry (fig. 2).

Free-floating sections of the LA and EDL were rinsed several times in phosphate-buffered saline (PBS), blocked 30 min in PBS containing sodium azide (0.1%), Triton X-100 (0.3%) and bovine serum albumin (0.2%) and then incubated at 4°C for approximately 36 h in the same blocking solution containing primary antibodies. The following primaries were used: a rabbit polyclonal antiserum directed against cow S100, a SC protein (Dako No. Z311, diluted 1:400) and two mouse monoclonal antibodies (MAbs), directed against the 165 kD neurofilament protein located in axons and the synaptic vesicle protein 2 (SV2) located in synaptic endings (Developmental Studies Hybridoma Bank MAbs 2H13 and SV2, respectively; both diluted 1:200). All subsequent steps were carried out at room temperature.

After incubation in primaries, muscle sections were rinsed in blocking solution and then incubated 1–2 h in blocking solution containing appropriate fluorescent or biotinylated secondary antisera. SCs were labeled with either rhodamine-labeled anti-rabbit secondary antisera (Cappel, 1:400) or fluorescein-labeled anti-rabbit secondary antisera (Vector, 1:150). Axons and their nerve terminals were labeled using either fluorescein-labeled anti-mouse, rat-adSORbed secondary antisera (Vector, 1:150) or visualized using Elite ABC peroxidase reagents (Vector) and a biotinylated, rat-adsorbed, horse anti-mouse secondary antisera (Vector, 1:200). HRP was reacted in a Tris buffer (0.05 M, pH = 7.2) containing 3,3-diaminobenzidine (DAB, 0.03%), nickel chloride (0.02%) and hydrogen peroxide (0.01%) and was stopped after 2 min by several rinses in PBS. AChRs were labeled with rhodamine-labeled α-bungarotoxin (Molecular Probes, 1:100) during incubation in the secondary antiserum.

Stained muscle sections were mounted on to gel-subbed slides and allowed to dry in the dark for at least 24 h at 4°C before coverslipping. Slides containing stained material were rinsed 10 min in water, dehydrated in a graded series of ethanol (2–3 min each) and cleared in Hemo-De (Fisher, orange-based clearing agent). D PX (Fluka) was used to coverslip fluorescent material and permount for DAB-reacted material. Slides containing fluorescently stained material were first counterstained with DAPI (0.1 μg/ml, 10 min to visualize cell nuclei before dehydration. After ≥ 48 h at 4°C, fluorescent stained material was stored at ~20°C. DAB-reacted material was stored at room temperature.

**Microscope and Statistical Analysis**

The described method of sealing and storing fluorescently stained muscle sections results in remarkably long-lived fluorescence, with little or no obvious fading for at least a year after the fluorescent tags were initially introduced into the tissue. Consequently, this method offers considerable flexibility during the analysis phase of an experiment. In the first set of stained LA and EDL muscle sections, in which TSCs, motor axons and their terminals were stained, we measured (1) the number of axonal inputs per junction, (2) the number of TSCs per junction and, with the aid of an ocular micrometer, (3) muscle fiber width. We sampled only those fibers for which all three measures could be taken. The second series of stained muscle sections were generated primarily to assess the effect of testosterone on endplate size and, for this purpose, we measured TSCs per junction and, with the aid of an ocular micrometer, (3) muscle fiber width. We sampled only those fibers for which all three measures could be taken. The second series of stained LA and EDL muscle sections, in which endplates were imaged, thresholded and their area estimated using NIH Image. Finally, in the third series (peroxidase stained) of LA and

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Fig. 1. Representative photomicrographs of LA NMJs from a control-treated, gonadally intact juvenile (28 days old) male rat stained with immune and nonimmune markers. A, B Same neuromuscular junction stained with mouse MAbs against a 165-kD neurofilament protein and the synaptic vesicle protein SV2, located in axons and nerve terminals, respectively, and a rabbit polyclonal antiserum against S100, located in TSCs. Axons and their nerve terminals (A) were visualized with the same fluorescein-labeled, anti-mouse secondary and TSCs (B) were visualized with a rhodamine-labeled, anti-rabbit secondary antiserum. Note that one axon (arrow in A) contributes to the NMJ and that two TSCs (arrows point to individual cell bodies) comprise this same NMJ. The rhodamine-labeled SCs are also somewhat visible in A under fluorescein illumination. In alternate sections from the same set of muscles, endplates were stained using rhodamine-labeled α-bungarotoxin (C), which binds to acetylcholine receptors, in combination with SC staining at the same NMJs using a fluorescein label. (D). Note that S100 is expressed by SCs generally, at both the NMJ and along preterminal axons. Two TSCs (arrows in D) are present at this NMJ. Note that the shape of the area occupied by the TSCs and their processes (D) appears to match that of its underlying endplate (C), as reported by numerous other investigators [e.g. Love and Thompson, 1998; Lubischer and Bebinger, 1999; O’Malley et al., 1999]. Scale bar = 30 µm.

EDL muscle sections, we measured the number of inputs per muscle fiber and muscle fiber width. For each section analyzed, muscle fibers were sampled across the entire section of the muscle (number of sampled LA muscle fibers reported in table 1) or in the case of the EDL, until 40 muscle fibers were sampled. Our samples included only those fibers with distinct margins and presenting en face junctions, which were innervated by axonal inputs that could be followed back into an intramuscular nerve branch and/or having TSCs whose cell bodies exhibited distinct boundaries (fig. 1) that could be readily resolved and counted. All measurements were based on 6–8 animals from 4–5 different litters. To evaluate the effect of testosterone treatment, separate one-way ANOVAs were carried out for each measure using software developed by Woodward et al. [1990] with directed post hoc comparisons.

In our preliminary analysis, we found that we could reliably distinguish the cell bodies of individual TSCs on the basis of S100 staining alone and that estimates of their number based only on S100 staining correlated well with the number of TSC nuclei detected by the additional label DAPI. As seen by others [e.g. Trachtenberg and Thompson, 1997; Love and Thompson, 1998; Lubischer and Bebinger, 1999], the nuclei of TSCs virtually fill the cell body and exhibit either dense S100 staining [also seen in the sciatic nerve: Jordan, unpubl. obs.] or are outlined by dense S100 staining with little inside (expect for an S100+ nucleolus). Even TSCs at the same junction can exhibit this different staining. While the reasons for this variability in S100 staining are not understood, we find that the number of TSCs at individual junctions can be reliably counted based on the pattern of S100 staining alone. This method was used for estimating TSC number, as done by others [Hirata et al., 1997; O’Malley et al., 1999].
**Fig. 2.** Representative photomicrographs of immunoperoxidase-stained LA NMJs from 27- to 28-day-old male rats left gonadally intact (A) or castrated and given testosterone (B) or no hormone (C) for three weeks prior to sacrifice. NMJs in LA muscles were stained as free floating sections using ABC peroxidase reagents and MAb against neurofilament and SV2 proteins. NMJs in A and C are examples of single innervation (arrows point to single axons which exclusively innervate individual muscle fibers), whereas the NMJ in B is an example of multiple innervation. Note that in B, two axons (arrows) converge to form an apparently unitary synaptic arbor that innervate a single muscle fiber. Testosterone treatment during synapse elimination have several morphological effects on LA NMJs: in addition to preventing the loss of multiple innervation, testosterone also markedly increases the size of fibers and their junctions. This increase in fiber and junctional size is associated with an increase in the number of TSCs present at each junction and appears to be independent of the number of inputs to each fiber (fig. 3, 4). Scale bar = 30 μm.

**Table 1.** Estimates of the percentage (mean ± SEM) of multiply innervated (% MI) LA muscle fibers based on fluorescent staining of neurofilaments in axons (left column) or SC ensheathment around axons without neurofilament staining (middle column), or peroxidase staining of neurofilament in axons (right most column)

<table>
<thead>
<tr>
<th></th>
<th>Fluorescent ICC neurofilament (+SV2/S100)</th>
<th>Fluorescent ICC S100 (+AChRs)</th>
<th>Peroxidase ICC neurofilament (+SV2)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>% MI sampled fibers</td>
<td>% MI sampled fibers</td>
<td>% MI sampled fibers</td>
</tr>
<tr>
<td>Sham + bl</td>
<td>22.0 ± 2.77</td>
<td>14.5 ± 0.90</td>
<td>20.47 ± 2.18</td>
</tr>
<tr>
<td>Cast + T</td>
<td>50.2 ± 2.26*</td>
<td>49.4 ± 2.92*</td>
<td>42.6 ± 5.09*</td>
</tr>
<tr>
<td>Cast + bl</td>
<td>21.4 ± 2.73</td>
<td>16.7 ± 2.13</td>
<td>17.9 ± 3.39</td>
</tr>
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</table>

Each set of estimates was based on alternate sections from the same muscles (mean ± SEM number of sampled muscle fibers/muscle based on n = 6–8 animals/group).

*p < 0.001, significantly different from shams or blank-treated castrates based on one-way ANOVAs. Sham + bl = control-treated shams; cast + T = testosterone-treated castrates, cast + bl = control-treated castrates.

**Results**

*Fluorescent or Peroxidase Labeling of NMJs Yields Comparable Estimates of Multiple Innervation*

The effect of testosterone on synapse elimination in the LA has previously been demonstrated using only the tetranitroblue tetrazolium (TNBT) staining method [Jordan et al., 1989] and not immunocytochemistry (ICC). Thus, our first objective was to determine whether we could detect an effect of androgen treatment on synapse elimination in the LA using ICC. Because fluorescence ICC is typically less sensitive than peroxidase ICC when using a compound microscope, we first simply compared estimates of multiple innervation in the LA derived from the three different staining methods. We found good correspondence between the estimates of multiple innervation on both the LA (table 1) and the EDL (data not shown). Testosterone treatment significantly (p < 0.001) increased the percentage of LA muscle fibers that are multiply innervated in all three sets of stained muscle sections. These results indicate that, based on our current staining and storage methods, fluorescent and peroxidase ICC allow comparable detection of neuromuscular inputs in juvenile muscles, as does the TNBT staining method. It is also noteworthy that staining either the neurofilament inside axons or the glial ensheathment outside axons leads...
Table 2. Mean (± SEM) LA and EDL muscle fiber width (µm) in alternate sections from the same muscles subjected to fluorescent or peroxidase ICC to stain neurofilament and SV2, axonal and synaptic vesicle proteins, respectively

<table>
<thead>
<tr>
<th></th>
<th>LA¹ fluorescent ICC</th>
<th>LA¹ peroxidase ICC</th>
<th>EDL² fluorescent ICC</th>
<th>EDL² peroxidase ICC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham + bl</td>
<td>14.2 ± 0.78</td>
<td>12.5 ± 0.12</td>
<td>20.1 ± 0.31</td>
<td>16.0 ± 0.62</td>
</tr>
<tr>
<td>Cast + T</td>
<td>19.2 ± 0.25*</td>
<td>16.5 ± 0.61*</td>
<td>20.9 ± 0.44</td>
<td>16.9 ± 0.69</td>
</tr>
<tr>
<td>Cast + bl</td>
<td>14.6 ± 0.40</td>
<td>12.4 ± 0.71</td>
<td>19.4 ± 0.37</td>
<td>15.4 ± 0.55</td>
</tr>
</tbody>
</table>

* p < 0.001, significantly different from shams or blank-treated castrates based on one-way ANOVAs; sham + bl = control-treated shams; cast + T = testosterone-treated castrates; cast + bl = control-treated castrates.

¹ Number of sampled LA muscle fibers reported in table 1.
² Number of sampled EDL muscle fibers = 40.

Testosterone Treatment during Synapse Elimination Increases the Size of LA Neuromuscular Junctions and the Number of TSCs

While our present data confirm previous results [Jordan et al., 1989a, 1995], present evidence also indicates that testosterone treatment during synapse elimination in the LA also increases the size of NMJs and the number of TSCs at NMJs.

One-way ANOVAs revealed significant main effects of treatment on all four measures in the LA (percent multiple innervation, muscle fiber width, endplate area, and number of TSCs per junction: p < 0.001). Post hoc comparisons indicate that LA muscles at P27–28 from castrates treated with testosterone for 3 weeks contain a significantly higher percentage of multiply innervated fibers, and have significantly larger muscle fibers and endplates and significantly more TSCs per junction than LA muscles from either blank-treated shams (gonadally intact) or blank-treated castrates (p < 0.001, fig. 3). Consistent with previous data [Jordan et al., 1989a, 1995], no differences (p > 0.05) were found in LA muscles from control animals (blank-treated shams and castrates). Moreover, the three estimates of multiple innervation, the two estimates of muscle fiber size and the two estimates of TSC number show the same group differences. In short, testosterone treatment during synapse elimination in LA muscles increases the amount of multiple innervation by about two-fold, the number of TSCs per LA junction by one, and the size of muscle fibers and endplates by about 25–30%.

Testosterone treatment had no effect on any measures (multiple innervation, muscle fiber width and TSC number) taken in the EDL. As reported for adult muscles [Lüscher and Bebinger, 1999], we found that juvenile EDL junctions had more TSCs than did juvenile LA junctions (3.44 ± 0.09 TSCs per EDL junction vs. 1.93 ± 0.05 TSCs per LA junction).

to similar estimates of the number of axons that innervate individual muscle fibers. However, estimates of multiple innervation are lower in testosterone-treated LA than the levels previously reported [e.g. Jordan et al., 1989a, 1995]. One possible explanation is that the present use of capsules for delivering testosterone, rather than daily injections as done previously, may be less efficacious at quickly increasing androgen titers to spare synapses already in the process of being eliminated.

The effect of testosterone treatment was also confirmed by weighing seminal vesicles, a convenient bioassay for androgen levels. As expected, seminal vesicles were heaviest in testosterone-treated castrates (values presented throughout as mean ± SEM: 357 ± 12.6 mg), intermediate in sham castrates (21.8 ± 1.0 mg) and lightest in blank-treated castrates (10.4 ± 0.48 mg), confirming the efficacy of castration and testosterone treatment, which effectively raises androgen titers above normal levels for this age. While there were no group differences in body weight at the beginning of treatment (P7), testosterone treatment also increased overall body weights (data not shown).

We did detect one difference between the fluorescent and peroxidase-stained muscle sections: both LA and EDL muscle fibers are consistently smaller (by about 15–20%) in the HRP condition (table 2). Since muscle sections were exposed to the same buffers and fixative, we suspect that some aspect of the final DAB reaction causes significant tissue shrinkage. However, in both staining conditions, the same group differences were observed, with testosterone treatment influencing the size of LA but not EDL fibers.
TSCs per LA junction; estimates based on muscle sections from blank-treated sham castrates in which SCs, motor axons, and nerve terminals were visualized using fluorescent markers). This difference is correlated with a difference in muscle fiber size (and presumably also endplate size). In gonadally intact juveniles (blank-treated shams), EDL fibers are larger than LA fibers (table 2).

While our results demonstrate that testosterone increases the number of TSCs at LA junctions, as testosterone maintains multiple innervation, it does not reveal whether the effect of testosterone on TSCs is specific to the effect of testosterone on multiple innervation. In other words, does testosterone increase the number of TSCs on only those fibers for which synapses have been spared, i.e., on multiply innervated fibers? To answer this question, we carried out additional two-way ANOVAs with one repeated measure, singly versus multiply innervated muscle fibers.

We predicted that if the effect of testosterone on the number of TSCs was specific to its effect on synapse maintenance, then the testosterone-induced increase in TSC number would be evident on only multiply and not singly innervated fibers. However, we found that testosterone significantly increased the number of TSCs on both singly and multiply innervated fibers (p < 0.001; fig. 4). In addition, testosterone also significantly increased the size of both singly and multiply innervated fibers and their junctions (p < 0.001; fig. 4). These results suggest that the number of TSCs during synapse elimination is related to the size of muscle fibers and their junctions and not the number of axonal inputs.

In addition to the significant main effect of treatment (as described for results based on one-way ANOVAs), the two-way, repeated-measures ANOVA also revealed a significant main effect of innervation (p < 0.005) on the number of TSCs per junction. Multiply innervated fibers tend to have more TSCs at their junctions than singly innervated fibers (fig. 4). No significant interaction was found. In parallel, the size of muscle fibers and their endplates were also significantly influenced by innervation (p < 0.005), with multiply innervated fibers tending to be larger and have larger endplates than singly innervated fibers (fig. 4). Post hoc comparisons revealed that multiply innervated fibers in testosterone-treated muscles tend...
Fig. 4. Three measures of neuromuscular morphology for singly versus multiply innervated LA muscle fibers from control-treated, gonadally intact males (sham + bl), testosterone-treated castrates (cast + T) and control-treated castrates (cast + bl) 27–28 days old. Values plotted are mean ± SEM. Testosterone treatment significantly increased all three measures on both singly and multiply innervated fibers compared to control-treated muscles from either gonadally intact or castrated males (p < 0.001). These data indicate that the androgen-induced increase in TSC number is related to fiber and junctional size and not to the androgenic sparing of synapses on multiply innervated fibers. A TSCs. B Muscle fiber width. C Endplate area.

to be larger (p = 0.052) and have larger junctions (p < 0.033) with more TSCs (p < 0.017) than singly innervated fibers from the same testosterone-treated muscles (fig. 4). Similar differences were observed in control-treated LA muscles, suggesting that the number of inputs may regulate or be regulated by the size of fibers and their junctions and, in turn, influence the number of TSCs.

Discussion

Previous work has shown that testosterone treatment during synapse elimination in the LA can permanently prevent the loss of synapses in this muscle. Hence, testosterone is a potent stabilizing factor for developing synapses in the LA. TSCs, which cap the neuromuscular junction, have also been implicated in the control of synaptic stability [Trachtenberg and Thompson, 1997] and may play a decisive role in the selective stabilization of synapses during synapse elimination. In this study, we began to investigate the possible role of TSCs in the effect of testosterone on synaptic stability by determining whether testosterone influenced their number. Male rats were castrated or sham gonadectomized at P7 and given capsules containing either testosterone or nothing. After 3 weeks of treatment (P27–28), NMJs in the LA and EDL muscles were stained using immune and nonimmune markers.

Confirming previous results based on TNBT staining and electrophysiological methods [Jordan et al., 1989a, 1992], we again found that testosterone partially prevents synapse elimination in the LA, maintaining multiple innervation that would ordinarily be eliminated. Normally the pattern of innervation in the LA goes from complete multiple innervation at P7 down to about 30% multiple innervation by P28 [Jordan et al., 1988]. Treatment with androgens, such as testosterone or dihydrotestosterone, markedly slows synapse loss, causing 60–70% of LA fibers to remain multiply innervated [Jordan et al., 1995]. On the other hand, estrogenic metabolites of testosterone have no sparing effect on LA synapses during synapse elimination [Jordan et al., 1995], marking it clear that androgen receptors and not estrogen receptors mediate the effect of testosterone on synapse elimination. Once multiple innervation is spared by androgen during synapse elimination, it remains indefinitely independent of androgens [Jordan et al., 1989b; Lubischer et al., 1992]. Testosterone also increases the size of individual LA muscle fibers [Jordan et al., 1989a, 1995; present results]. The present study demonstrates for the first time that testosterone treatment, which perturbs synapse elimination,
also increases the number of TSCs per junction and the size of LA junctions (fig. 3). These data raise the question of whether the effect of testosterone on TSC number (or the size of LA fibers and their junctions) is responsible for its effect on multiple innervation. To answer this question, we categorized LA fibers into singly versus multiply innervated and asked whether the effect of exogenous testosterone on TSC number, fiber size, and/or junctional size is exclusive to multiply innervated fibers. We found that testosterone treatment significantly increased all three measures on both singly and multiply innervated fibers (fig. 4), confirming previous results on fiber size [Lubischer et al., 1992]. These results suggest that while testosterone is capable of sparing synapses on only some fibers, it increases the number of TSCs and the size of LA fibers and their junctions on most, if not all, LA fibers. Importantly, testosterone increases the number of TSCs and fiber and junctional size on fibers for which it fails to spare synapses.

We should point out that depriving the developing LA of testicular androgens had no effect on any of the measures taken at P27–28, since no differences were observed between control-treated castrates and sham castrates (fig. 3, 4). This result is consistent with prior observations, indicating that while endogenous androgens during the prepubertal and pubertal stages of development have striking effects on the time course of synapse elimination, delaying the onset by days and the completion by an entire month, there were no differences in either the pattern of innervation or size of fibers in LA muscles at P28 [Jordan et al., 1989a, b]. While the effects of testosterone treatment on developing LA synapses clearly reflect a super-physiological action of androgens in juvenile animals, treating young animals with apparently nontoxic doses of testosterone nonetheless offers a powerful approach for identifying basic mechanisms critically involved in synaptic maintenance and stabilization.

Although information does not yet exist about the developmental time course of TSC number during synapse elimination in the LA, data from the rat EDL and soleus suggest that TSC coverage is minimal at the beginning of synapse elimination with nerve terminals covered largely by TSC processes and sometimes by a cell body [Love and Thompson, 1998]. As synapse elimination ensues, TSCs steadily increase in number. Data from the normal, unmanipulated soleus also suggest that TSCs accumulate at individual junctions, independent of the number of axonal inputs, since singly and multiply innervated fibers have equivalent numbers of TSCs during synapse elimination [Hirata et al., 1997]. While we do not yet know why testosterone stabilizes multiple synapses on only some LA fibers, present results suggest that the effect of testosterone on TSC number is not specific to its effect on synapse maintenance. Rather, it appears more likely that the number of TSCs in androgen-treated muscles is related to the size of muscle fibers and/or their NMJ (fig. 4). This correlation between muscle fiber and/or junctional size and the number of TSCs has been reported for the adult LA and for other (androgen-insensitive) muscles [Love and Thompson, 1998; Lubischer and Bebinger, 1999]. In short, the number of TSCs appears to be unrelated to the stabilization of synapses by testosterone. This tells us nothing about whether other, nonandrogenic influences on synapse elimination act through altering TSC number.

Despite the fact that testosterone increased the number of TSCs on both singly and multiply innervated fibers, the amount of innervation did generally correlate with the number of TSCs, since in all treatment conditions, multiply innervated fibers tend to have more TSCs than singly innervated fibers (fig. 4). However, multiply innervated fibers are also generally larger and have larger junctions. In light of the fact that testosterone increases the number of TSCs on singly innervated fibers without sparing synapses, these data also suggest that the salient relationship is between the number of TSCs, the size of fibers and/or their junctions. Whereas the present study does not address the causality of this relationship, prior evidence suggests that the number of motoneurons innervating LA fibers influences muscle fiber size and not vice versa [Lubischer et al., 1992]. Hence, more motoneurons innervating some fibers may lead to their increased growth, which influences the size of their endplates and the number of TSCs. Motoneurons could influence muscle fibers in this way through either activity or the release of trophic factors.

While evidence so far suggests that the number of TSCs may not be related to the number of inputs in any simple manner [Hirata et al., 1997; Love and Thompson, 1998; present results], TSC number may nonetheless be an important factor in maintaining synapses. The stable maintenance of a neuromuscular synapse, whether it is formed by one or more axons, may require complete coverage of that synapse [Trachtenberg and Thompson, 1997]. The lawful relationship between the size of junctions and the number of TSCs [Love and Thompson, 1998; Lubischer and Bebinger, 1999; Herrera et al., 2000; present results] suggests that each TSC can cover only a limited amount of synaptic membrane. Thus, as the synapse expands its territory, independent of the number of

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axons forming that synapse, more TSCs may be required to adequately cover the synapse. Since testosterone increases the size of the junctions on both singly and multiply innervated fibers, the effect of testosterone on TSCs may reflect the need for more TSCs to cover a larger synaptic area.

Apart from their number, compelling evidence suggests that TSCs per se play a crucial role in ensuring that at least one synaptic input per muscle fiber is stably maintained. If TSCs are induced to migrate away from the endplate during synapse elimination, nerve terminals regress and are eventually eliminated [Trachtenberg and Thompson, 1997]. Not only do these data suggest that glial coverage is an important aspect of synaptic stability, but also that the stability of synapses depends on the stability of TSCs. In addition, while neuromuscular synapses can apparently form in the absence of SCs, they are only transiently maintained and quickly eliminated [Lin et al., 2000]. Observations in frog pectoral muscles also suggest that innervation of primary myotubes may occur without the aid of SCs and that the primary role of SCs is to cover and then dictate the growth of early contacts [Herrera et al., 2000]. Finally, recent in vitro evidence suggests that astrocytes, which ensheathe synapses in the central nervous system, also exert a stabilizing influence on synapses: synapses induced to form by astrocyte-conditioned medium are not maintained when the conditioned medium is removed [Ullian et al., 2001]. Hence, glia actively participate in the stabilization of synapses, but TSCs do not seem to be directly responsible for the stabilizing influence of testosterone on LA synapses.

Testosterone treatment did increase the number of TSCs, even if the extent of TSC addition appeared to be unrelated to the number of synaptic inputs maintained. This result raises the question of where testosterone acts, directly or indirectly, to increase the number of TSCs. Among the likely targets are LA motoneurons, located in the spinal nucleus of the bulbocavernosus, and the LA muscle itself. Data based on steroid autoradiography and androgen receptor ICC suggest that both LA motoneurons and LA muscle fibers have androgen receptors by the onset of synapse elimination in the LA [Jordan et al., 1991, 1997]. Thus, testosterone could act at one or both of these sites to influence TSC number. Alternatively, it might act directly on TSCs to increase their number. While an attractive idea, preliminary evidence suggests that TSCs in the adult LA do not express androgen receptors [Jordan, unpubl. obs.]. The possibility remains that LA TSCs transiently express androgen receptors during synapse elimination and thus are direct targets for androgens.

The two likely cellular mechanisms mediating the increase in TSC number are cell migration and cell division, since both have been shown to contribute to the increase in the number of TSCs during ontogeny of NMJs in the rat soleus and EDL [Love and Thompson, 1998]. SCs in each of these phases have distinct morphologies. Rounded cell nuclei on the preterminal axon segment just proximal to a muscle fiber are suggestive of cell migration, and apparent pairs of SC nuclei at the junction are suggestive of cell division [Love and Thompson, 1998]. While we observed both sorts of morphologies, suggesting that both cell migration and cell division contribute to the increased accumulation of TSCs in testosterone-treated muscles, we did not detect a difference in their incidence in testosterone-treated muscles compared to controls. An increase of about one TSC per junction, however, may not lead to a detectable increase in the incidence of such morphologies, particularly at a single time point near the end of synapse elimination in the LA.

In sum, we have shown that the androgenic sparing of synapses during synapse elimination is generally associated with an increase in the size of junctions and the number of TSCs per junction. Because the increase in junctional size and TSC number is also seen on singly innervated fibers, where testosterone fails to spare synapses, we conclude that the effect of testosterone on TSC number during synapse elimination is not related to its ability to spare synapses from elimination.

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