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Retention of Hormonal Sensitivity in Free Grafts of the Levator Ani Muscle

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The regenerating levator ani muscle of the rat is known to retain its sensitivity to the effects of testosterone. To determine whether hormonal sensitivity of the regenerating muscle is purely myogenic or requires some interaction with the pudendal nerve or the local humoral environment, levator ani muscles were freely grafted in place of the soleus or extensor digitorum longus muscles in normal, castrated, or testosterone propionate-treated rats. Grafts into the bed of the soleus were unsatisfactory due to poor reinnervation. Levator ani muscles grafted into the bed of the extensor showed a sensitivity to testosterone as demonstrated by gross wet weight, cross-sectional area of the muscle fibers, and twitch and tetanic tensions. Neither histochemical analysis nor contraction times were useful in distinguishing between hormonal groups. It is concluded that hormonal sensitivity of the regenerating levator ani muscle is principally, if not entirely, myogenic.

INTRODUCTION

Recent work showed that the levator ani muscle in the rat retains its high sensitivity to testosterone when regenerating *in situ* after total crushing (8). Hormonal effects were demonstrated in several parameters. e.g., wet weight, muscle fiber area, contractile tension, and certain of the measured contractile speeds. From that experiment it could not be determined whether the hormonal sensitivity was purely myogenic or whether

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some interaction between the regenerating muscle and the pudendal nerves and/or local humoral environment was necessary to bring about the full sensitivity to testosterone. This problem was attacked by taking the intact levator ani from its normal site and freely autografting it into the bed of a limb muscle. With this experimental model it was possible both to produce a massive regenerative response in the levator ani and to remove the regenerating muscle from its local neural and humoral environment.

MATERIALS AND METHODS

These experiments were carried out on 136 male rats of the Wistar and Sprague–Dawley (Charles River) strains. The former strain was used for analysis of contractile properties in Prague and the latter for quantitative morphological analysis in Michigan.

The rats were anesthetized with ether and the levator ani muscle was removed through a ventral scrotal incision. The intact muscle was then freely grafted into the bed of either the soleus or the extensor digitorum longus muscle, after removal of that muscle. The levator grafts fit quite well into the foreign beds, but after suturing of both ends of a graft to the proximal and distal tendon stump of the graft site the transplanted levator ani was under somewhat less resting tension than would be an orthotopic soleus or extensor digitorum longus graft. The reduced resting tension did not interfere with final integration of the graft with its functional bed. It is likely that two factors—(i) the breakdown and subsequent reorganization of the graft and (ii) the growth of the leg pulling against the early nongrowing graft—work to produce ultimately a normal degree of tension on the graft. The grafting procedure resulted in a muscle with a thin rim of surviving muscle fibers and a massive population of regenerating muscle fibers throughout the rest of the graft.

Hormonal Status of Rats. The rats were divided into three different hormonal groups: (i) normal hormonal status, (ii) bilateral castration, and (iii) added testosterone. Testosterone was administered in the form of implants of Dow–Corning Silastic tubing (0.32 cm, outer diameter; 0.16 cm, inner diameter; 62.5 mm, length), filled with testosterone propionate (U.S. Biochemical Corp.). These tubes, sealed on either end with No. 382 medical grade elastomer (Silastic, from Dow–Corning), were implanted beneath the skin of the back at the time of muscle transplantation. These implants continuously release 2.5 mg hormone per day (1).

Histological Analysis. Fourteen levator ani muscles grafted in place of both the soleus and extensor digitorum longus were removed for histiological analysis at various early postoperative intervals to verify that regeneration of muscle was taking place in the grafts. Morphometric analysis was carried out on 30 muscles in five groups of rats. All rats were from

the same initial batch. They were of similar age and weight at the start and were kept under the same conditions of housing and feeding throughout the experiment. The experiment was begun when the rats were 1 month old and it lasted 60 days. The first group was a normal control. The animals remained untreated throughout the experiment. Three groups, with six animals per group, consisted of levator ani muscles grafted in place of the extensor digitorum longus in normal, castrated, and testosterone-treated rats. In another group, the levator ani was grafted in place of the soleus muscle in six rats. Histological examination was made on the other grafts from both the contractile and histochemical series. Muscles were fixed in Bouin's fluid, sectioned at 7 μm, and stained with Ehrlich's hematoxylin and eosin. For quantitative analysis, cross sections of grafts were projected onto a wall, and outlines of 100 muscle fibers per section were traced on paper. Cross-sectional areas of these muscle fibers were determined by planimetry.

Histochemical Analysis. Histochemical study proved not to be useful in the analysis of hormonal effects on the regenerating levator ani (8), but on selected grafts histochemical analysis established the degree of integration of long-term grafts with their heterotopic sites in the leg. Grafts were frozen in liquid nitrogen, and frozen sections were stained for succinic dehydrogenase (14) and myofibrillar ATPase (7, 15).

Analysis of Contractile Properties. Contractile properties were measured in 68 animals. In one group, 12 control levator ani muscles were tested in normal 1-, 2-, and 3-month-old rats. In another group, the levator was grafted in place of the soleus in six 2-month-old rats, and contractile function was analyzed 30 days later.

The main experimental series (levator grafted in place of extensor digitorum longus) was divided into two subgroups on the basis of previous work on hormonal effects on the regenerating levator ani (8). In the first subgroup, regeneration of grafted muscles was compared between normal and testosterone-treated animals. For this group, 1-month-old rats were used because of the low levels of circulating testosterone at this age. Contractile properties were measured 7 and 14 days postoperatively because at these times the greatest differences were noted between normal and testosterone-treated rats. The second subgroup consisted of a comparison of levator grafts in normal and castrated rats, which were 2 months old at the time of the operation. That age was chosen because the rats normally have very high levels of circulating testosterone (13). Grafts were analyzed at 14 and 30 days because castration effects take longer to become distinct than do the effects of added testosterone. In all rats bearing grafts, the contralateral normal soleus or extensor digitorum longus muscle was analyzed for a control.

For contractile analysis, the grafts and normal control muscles were removed from rats anesthetized with ether, and ligatures were tightly tied to their newly formed tendons of origin and insertion. The muscles were then placed in an oxygenated culture medium (149.8 mM Na^+, 5.0 mM K^+, 2.0 mM Ca^{2+}, 148.0 mM Cl^-, 12.0 mM HCO_3^-, 1.0 mM H_2PO_4 and 11.0 mM glucose, *p*H 7.2) to which 0.01 M tubocurarine had been added and equilibrated in this medium for 10 min in a 36°C chamber. The optimal resting tension for isometric recording was then determined by direct stimulation of the muscles with platinum electrodes. The following contractile properties were recorded on an automated apparatus for measuring muscle contraction (16): twitch and tetanic tension, latency period (stimulus artifact to first mechanical response), contraction time (time to peak tension), and half relaxation time (from peak to one-half amplitude of the twitch).

RESULTS

The Levator Ani as a Free Graft. Histological sections demonstrated that the reactions to grafting of the levator ani are essentially the same as those of limb muscles. A thin peripheral rim of surviving muscle fibers surrounds a large central area in which all other muscle fibers undergo ischemic necrosis and then break down due to the actions of macrophages, which accompany the blood vessels that grow into the graft. Regeneration of new muscle fibers follows the breakdown of old ones (Fig. 1). Grossly, the grafts become well established in their new beds. A unique feature of levator ani grafts was a pronounced difference between the proximal and distal halves (Fig. 2). The proximal half was commonly quite bulky whereas the distal half was normally attenuated. The midline raphe of the levator served as a sharp line of demarcation between the two halves.

Soleus vs. Extensor Digitorum Longus as a Graft Site. Initially levator muscles were grafted into the beds of both the soleus and extensor digitorum longus muscles in normal rats. Grafts placed into the bed of the soleus were consistently less successful than grafts placed into the bed of the extensor. The mean weight of the former grafts was less than half the weight of the latter (Table 1), and in 30-day grafts the mean contractile strength was considerably less than that of the contralateral normal soleus (twitch tension of graft was 4.83 ± 0.7 vs. 19.52 ± 1.4 g for the normal soleus, and tetanic tension was 14.85 ± 1.3 vs. 167.62 ± 5.7 g, for the soleus). Nevertheless, the histochemical pattern of mature muscle fiber types was characteristic of that of the soleus and other muscles grafted into the bed of the soleus.

Histological and histochemical examination of these grafts revealed

FIG. 1. Longitudinal section through a 7-day graft of the levator ani muscle into the bed of the extensor digitorum longus. The original muscle fibers of the graft have broken down, and the graft contains thin bands of newly regenerating muscle fibers. Hematoxylin and eosin stain, ×000.

bundles of very thin muscles interspersed among fibers of more normal size (Fig. 3). The thin muscle fibers stained uniformly darkly for adenosine triphosphatase (ATPase) and SDH (succinate dehydrogenase) activity. Both the contraction time and half relaxation time of the grafts were significantly faster than those of the normal soleus. The accumulated evidence strongly suggested that the lack of success of levator grafts in place of the soleus was due to incomplete innervation of the graft. Therefore subsequent experiments involved grafting of the levator into the bed of the extensor digitorum longus, which proved to be a very satisfactory site.

Hormonal Effects on the Levator Ani Grafted in Place of the Extensor Digitorum Longus. Two-month-old levator ani grafts demonstrated pronounced differences in response to altered hormonal environments. The ratio of wet muscle weight: body weight of levator grafts in testosterone-treated rats was almost twice as great as the ratio in castrated rats (Table 1). The ratio for grafts in normal rats was an intermediate value.

The same muscles were cross-sectioned and used for measurements of cross-sectional areas of muscle fibers. The mean values for muscle fibers in grafts of testosterone-treated rats were about twice that of those in

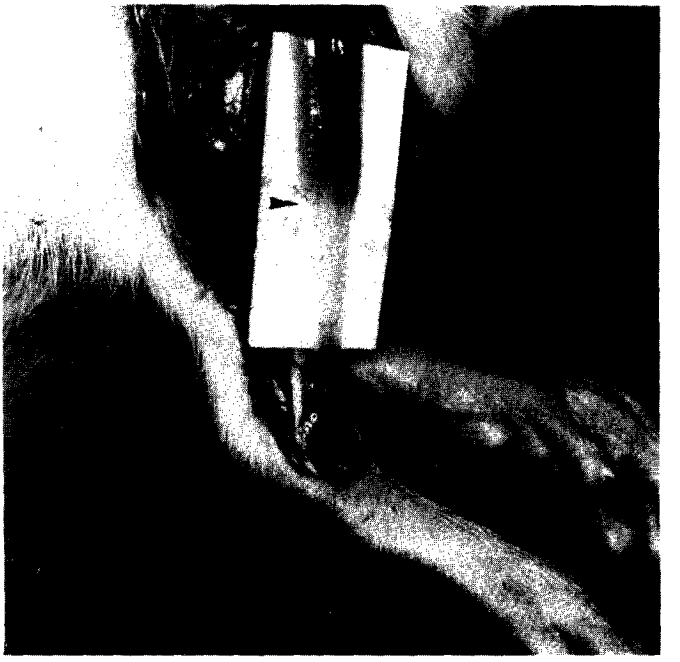

FIG. 2. Sixty-day grafts of the levator ani muscle into the bed of the extensor digitorum longus in a rat without any hormone treatment. The proximal half of the muscle is relatively massive. Distal to the level of the midline raphe of the levator ani muscle (arrow) the graft becomes abruptly attenuated and atrophic.

castrated rats (Table 2). A frequency distribution of muscle fiber areas reveals a clear-cut distinction between the hormone-treated and castrated groups (Fig. 4).

Compared with regeneration of the levator ani *in situ*, levator grafts in the three hormonal groups differed from one another to a lesser degree. In neither of the paired subgroups (normal vs. testosterone-treated and normal vs. castrated) was there significant differences in the various time parameters of contraction (latency period, contraction time, time constant of contraction, and half relaxation time) between grafts in normal and hormonally altered rats. A set of data comparing grafts between normal and testosterone-treated rats is given in Table 3.

The most pronounced hormonal effects on contractile properties were seen in the twitch and tetanic tensions of levator grafts in testosterone-treated rats (Fig. 5). By 14 days, the mean twitch tension of grafts in testosterone-treated rats was almost double that in normal grafts and 75% that of the contralateral normal extensor digitorum longus. The tetanic tensions showed similar, but less pronounced differences. As is typical of

TABLE 1

Weights of 60-Day Levator Ani (LA) Muscle Autografts in the Rat[a]

Graft site and hormonal treatment	Muscle weight (mg)	Muscle weight/body weight (mg/100 g)
Control LA *in situ* in normal rat	340.7 ± 14.0	91.0 ± 4.7
In soleus in normal rat	49.7 ± 5.5	12.1 ± 1.5
In EDL[b] in normal rat	117.6 ± 16.9	30.2 ± 4.0
In EDL in castrated rat	75.7 ± 6.5	22.8 ± 2.0
In EDL in testosterone-treated rat	129.0 ± 13.2	40.7 ± 3.5

[a] Means and standard errors of six rats per group.
[b] Extensor digitorum longus.

immature free muscle grafts and early regenerating muscle, the twitch–tetanus ratios of both hormonal groups were much more narrow than those of the control extensor digitorum longus muscles.

In the subgroup comparing grafts in castrated vs. normal rats, the mean

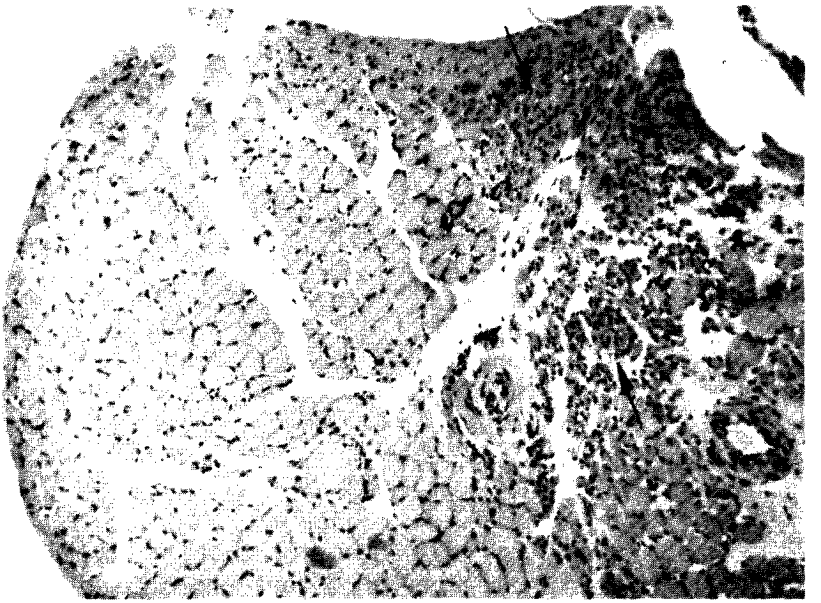

FIG. 3. Cross section through a 60-day graft of the levator ani muscle into the bed of the soleus in a rat without any hormone treatment. Although much of the graft looks good, there is a significant area (arrows) in which the regenerating muscle fibers are atrophic, presumably due to a lack of innervation. Hematoxylin and eosin stain, ×00.

TABLE 2

Mean Cross-Sectional Areas of Muscle Fibers from 60-Day Grafts of Levator Ani Muscles in Place of the Extensor Digitorum Longus

Hormone treatment	No. of muscles	Mean cross-sectional area ± SE (μm^2)
Normal	6	671 ± 78
Castrated	6	552 ± 53
Testosterone propionate	6	1116 ± 70

twitch tensions of grafts in castrated animals decreased from 10.6 ± 1.8 to 5.0 ± 0.8 g between 14 and 30 days postoperatively (Fig. 6). For unknown reasons the mean twitch tension of the normal group also decreased from 9.4 ± 0.6 to 6.4 ± 0.7 g from 14 to 30 days. This was correlated with considerably lower than usual weights of the normal grafts in this series and may be a reflection of the variabililty in results that can occur in long-term muscle grafts.

DISCUSSION

The main question underlying this investigation was whether the hormonal sensitivity of the regenerating levator ani muscle in the rat is purely myogenic or some interaction between the regenerating muscle fibers and the pudendal nerve and/or local humoral environment is required to elicit the full hormonal sensitivity of the regenerating muscle. The transplantation model was used for attacking this problem because previous work on the grafting of limb muscle showed that a high proportion of muscle fibers in a graft degenerate and subsequently regenerate (4) and also because grafting into the leg places the levator in a foreign neural and humoral environment.

The preliminary work on this experimental model demonstrated that muscle fiber grafts of the levator ani into the leg do, indeed, react like transplanted limb muscles by degenerating and then regenerating. Gutmann and Hanzlíková (9) previously showed that grafts of the levator ani into the sites of either the soleus or tibialis anterior muscles adapted to the graft site in both contractile and histochemical properties.

The results of these experiments leave little doubt that the regenerating levator ani muscle remains sensitive to the effects of testosterone when grafted into the leg. These effects are reflected in prominent differences between normal, castrated, and hormone-treated groups of rats with respect to (i) gross weights of the grafts, (ii) cross-sectional areas of

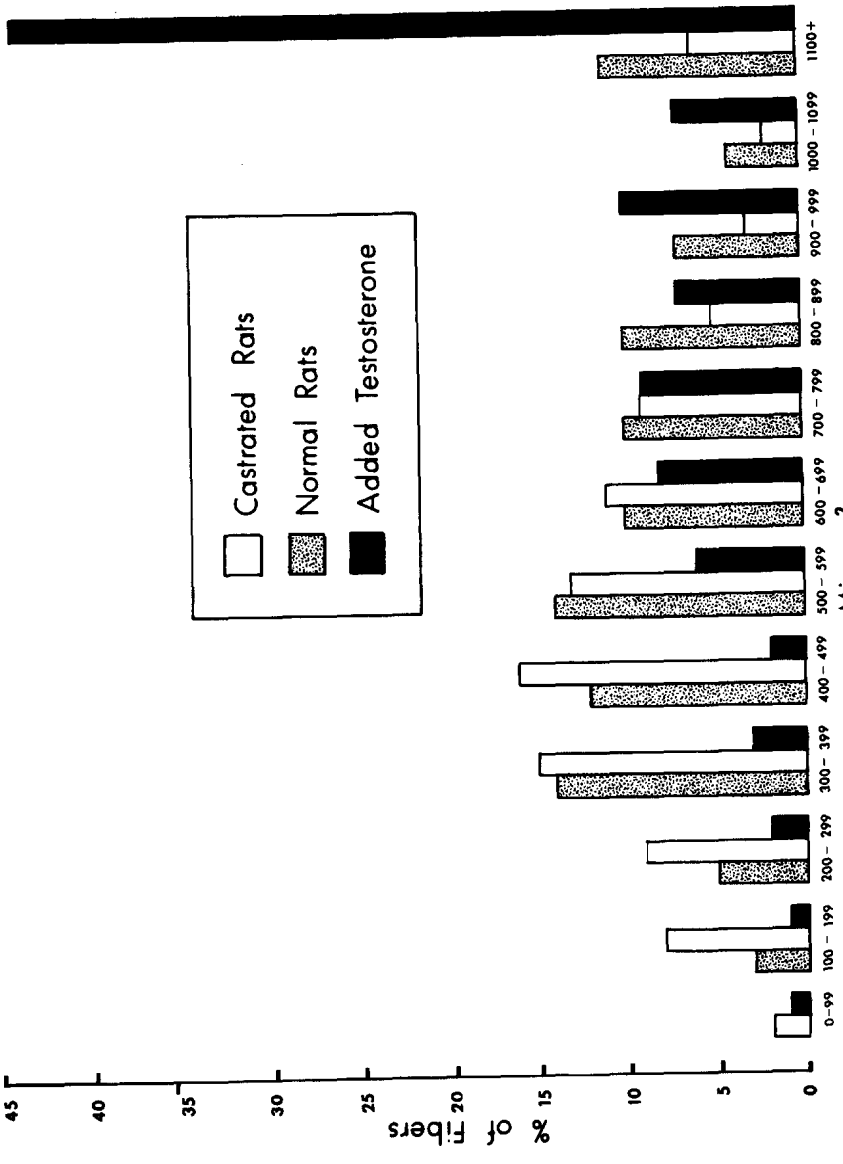

FIG. 4. Frequency distribution of cross-sectional areas of muscle fibers in 60-day levator ani grafts into the bed of the extensor digitorum longus (EDL) in normal, castrated, and testosterone-treated rats.

TABLE 3

Contractile Properties of 14-Day Levator Ani Muscles Grafted into the Extensor Digitorum Longus (EDL) in 1-Month-Old Rats

Hormone treatment	No. of rats	LP[a] (ms)	TCC (ms)	CT (ms)	HRT (ms)	Muscle weight (mg)	Rat weight (g)
Normal	6	2.9 ± 0.3	14.9 ± 0.6	25.5 ± 2.0	23.6 ± 2.3	41.8 ± 5.4	158 ± 11
Added testosterone propionate	6	2.9 ± 0.2	14.6 ± 0.9	23.8 ± 1.0	20.9 ± 1.1	53.8 ± 2.4	175 ± 10
Control EDL muscle	6	2.7 ± 0.1	7.9 ± 0.3	12.4 ± 0.2	7.5 ± 0.5	84.5 ± 6.5	175 ± 10

[a] Abbreviations: LP—latency period, TCC—time constant of contraction, CT—contraction time, HRT—half relaxation time.

muscle fibers within the grafts, and (iii) twitch and tetanic tensions of the grafts. The various time parameters of the isometric twitch did not prove to be useful in discriminating between experimental groups. This is in marked contrast to our earlier study (8) in which added testosterone

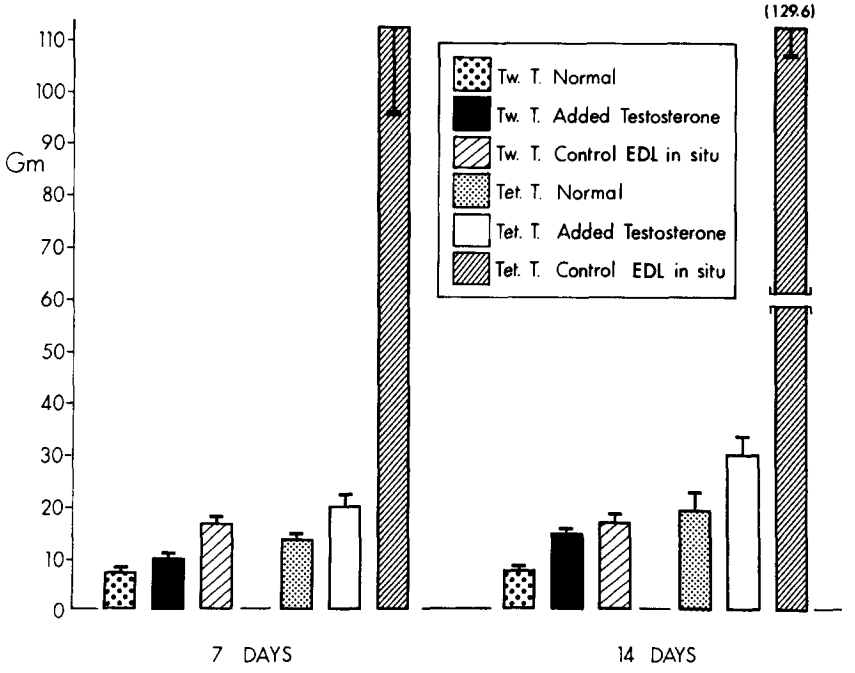

FIG. 5. Twitch and tetanic tensions of levator ani grafts into the bed of the extensor digitorum longus (EDL) muscle in normal and testosterone-treated rats. The rats were 1 month old when the muscles were grafted. Days on the graph refer to days after grafting.

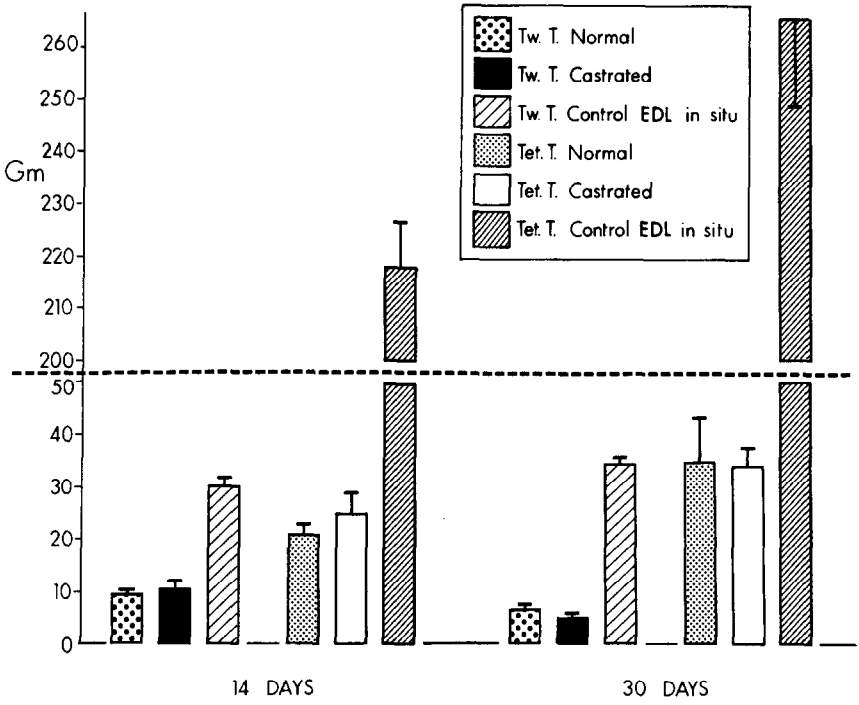

FIG. 6. Twitch and tetanic tensions of levator ani grafts into the bed of the extensor digitorum longus (EDL) muscle in normal and castrated rats. These rats were 2 months old at the time of grafting. Days on the graph refer to days after grafting.

speeded and castration slowed contraction speeds of crushed levator ani muscles *in situ*. In both the *in situ* crushing and the present grafting experiments, added testosterone significantly increased the contractile strength of the regenerating muscles early during the regenerative process whereas castration effects took longer to appear.

The present grafting experiments allow one to rule out both specific interactions with the pudendal nerve and other factors peculiar to the scrotal environment as essential to the development of hormonal sensitivity of the regenerating levator ani muscle. The lack of specificity of the pudendal nerve in maintaining the hormonal sensitivity of the intact levator ani was earlier shown by Hanzlíková and Gutmann (10), who cross-innervated the levator with the tibial nerve and found that the muscle still responded to testosterone and to castration. Additional evidence supporting the absence of specific properties of the pudendal nerve in promoting hormonal sensitivity was obtained by Hanzlíková and Gutmann (11), who grafted the soleus muscle into the bed of the levator ani. Although the soleus was transformed into a fast muscle, it did not acquire hormonal sensitivity.

An unpublished experiment showed as well that unilateral castration does not produce any detectable changes in the corresponding half of the levator ani muscle *in situ*.

Although a specific interaction between the functioning pudendal nerve and the muscle can now be discounted as critical for hormonal sensitivity of both the intact and regenerating levator ani, the experiments done to date cannot yet eliminate the possibility that hormonal sensitivity in the levator ani might require some nonspecific interaction between the muscle and any type of nerve. Two experimental findings, however, suggest that this is not the case. First, in the present experiments a pronounced increase in both twitch and tetanic tensions of levator grafts in testosterone-treated rats was seen as early as 7 days postgrafting. At that time the first nerve fibers are just entering the graft and motor end-plates were not yet formed. Second, an unpublished experiment in which levator ani muscles were grafted into 18 denervated legs showed that 1 month after grafting the noninnervated grafts in testosterone-treated rats were 80% heavier than noninnervated grafts in castrated rats. In addition, the demonstrations of testosterone binding to the cytosol receptor (12) and the ability of testosterone to affect protein synthesis of the levator ani *in vitro* (2, 3) provide further support for the independence of this effect from nerves.

The functional environment of the leg appears to exert a significant effect upon the levator ani grafts, which undergo a relative hypertorphy when placed into the bed of the extensor digitorum longus muscle. There is considerable variation in the cross-sectional diameter of regenerated muscle fibers within levator ani grafts, but the mean cross-sectional area of muscle fibers of grafts in both normal and testosterone-treated rats is larger than that of fibers in the normal levator ani of rats of the same age, and in some fascicles, the muscle fibers of grafts are greatly hypertrophied. Unfortunately, little is known about the ongoing function of the levator ani *in situ*, so that the basis for the differences noted above cannot at present be resolved.

Changes in the hormonal status of the rats affects the levator ani muscle regenerating in the leg differently from the muscle regenerating *in situ*. *In situ*, castration drastically reduced the size of the regenerating muscles whereas testosterone administration resulted in only a relatively small increase in size (8). In the grafted levator ani, however, the effects of castration were less marked and the effects of added testosterone were more pronounced. If it can be demonstrated that the contractile activity of the levator ani grafted into the leg is, indeed, greater than that of the levator *in situ*, it would lend support to the concept that increased function may have a potentiating effect upon the hormonal effects.

The incidental observation that the proximal half of levator ani grafts

is large and the distal half is attenuated (Fig. 2) is a complicating factor in the analysis of contractile properties of the entire muscle graft, but it could also prove useful as an experimental model. The reason for this unusual configuration seems to be that the proximal half of the muscle is innervated, whereas the half distal to the midline raphe of the levator does not become innervated. Further work would have to be done to validate this point, but indirect evidence (small muscle fiber diameter and lack of differentiation of muscle fiber types ith ATPase staining) points to the lack of innervation of the distal segment. If it could be demonstrated that all muscle fibers of the distal segments of these grafts are consistently denervated, it would then be possible to use levator grafts to compare the differences between innervation and denervation on muscle fibers which are arranged in series in the same mechanical environment.

A final noteworthy observation is the relatively large size and contractile strength of levator ani grafts in testosterone-treated rats. Within 14 days after grafting the twitch tension of these grafts was already 75% that of the normal extensor digitorum longus muscle from the same rats. In contrast, the twitch tensions of mature orthotopic extensor grafts in rats are commonly in the range of 35 to 50% of normal (5, 6). With respect to the problem of free-muscle grafting, this result suggests that the ideal donor muscle, at least in males, would be a muscle that is sensitive to testosterone.

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