Androgen Control of Myofiber Number and Size in the Rat *Levator Ani* Muscle

Christine Tobin, Yolaine Joubert, Marie Claude Lebart

Institut des Neurosciences, Département de Neurobiologie des Signaux Intercellulaires, CNRS URA 1488, 7 quai Saint Bernard, 75252 Paris Cedex 05

Abstract

The rat *levator ani* muscle is characterized by a marked sexual dimorphism resulting from differences in the number (male 5 300 ± 687 vs female 473 ± 251) and the diameter (cross-sectional area: male 718.67 ± 147.76 μm^2 vs female 65.88 ± 21.73 μm^2) of the muscle fibers of both sexes. Temporal patterns of myofiber formation on one hand, and of myofiber enlargement on the other hand have been studied.

Myofibers differentiate from primary and secondary myotubes which can be distinguished based on their morphological characteristics. Ultrastructural study and count of myotubes show that sexual dimorphism appears at fetal days 19-20 (E19-E20) and seems to be related to the occurrence of secondary myotubes. Myotubes continue to form and differentiate during postnatal development, between D0 (day of birth) and D5. At this stage the male LA muscle contains numerous immature myotubes and replicating cells. In contrast, during the same period in female muscle, few immature myotubes and dividing cells are present. Muscle fiber formation is nearly completed in female muscle at D5 but only at D11 for the male muscle. Testosterone treatment of newborn females, evaluated 2 months later, induces a permanent increase in the number of muscle fibers but this effect is observed only if testosterone is given before D6. Correlations between the different stages of muscle fiber formation and the variations in plasma testosterone level have been discussed.

Myofibers enlargement has been evaluated by measurement of the fiber cross-sectional area. Cross-sectional area shows no significant difference on D15 between either sexes (male 34.31 ± 12.77 μm^2 vs female 39.49 ± 11.57 μm^2). The most striking observation is a sharp increase in the male muscle fiber cross-sectional area from D42 (onset of puberty) to D78 leading to a fiber cross-sectional area about 10-fold higher in males (male 718.67 ± 147.76 μ^2 vs female 65.88 ± 21.73 μm^2). This muscle hypertrophy is accompanied by a transient increase in satellite cell number between D42 and D47 and a subsequent increase in myonuclei number from D47 which persists at least until D78. Hence during this period, the myonuclei number has increased by about 50% in the male LA muscle, whereas it remains unchanged in the female. Testosterone treatment of 30-day-old male and female rats generates muscle fiber enlargement, transient satellite cell proliferation and myonuclei increase at the same rate in both sexes. Our investigations strongly suggest that during puberty endogenous testosterone regulates muscle fiber hypertrophy and controls the myonuclei number *via* satellite cell proliferation. Altogether, our results demonstrate that testosterone acts on development and growth of the LA muscle during two critical periods: the perinatal period and puberty.

Key words: *Levator ani* muscle, myofiber formation, puberty, testosterone.

BAM 3 (2): 121-131, 1993

In different vertebrate species some striated muscles are more developed in the male than in the female. Examples in amphibians are the laryngeal muscle in *Xenopus laevis* [30, 55, 38] and the hindlimb muscles in *Rana temporaria* [44, 59], in birds the syringeal muscle [36, 37] and in mammals the masseter in the guinea pig [57, 33], the perineal muscles in rats [65, 26, 10, 19] and mice [63, 64], the shoulder muscles in humans.

The more or less marked sexual dimorphism that these muscles acquire during development has been explained by the stimulatory action of androgens on their growth [48, 65, 23]. However, the way this regulation is exercised appears complex and is not yet fully understood.

The rat *levator ani* muscle (LA) is among the best known hormone-dependent muscles. In male rats, it constitutes along with the *bulbocavernosus* (BC) and the *ischiocavernosus* (IC) muscles the perineal complex. BC and LA muscles are implicated in penile reflexes [53, 66, 41]. However, a recent work has reported that LA is not actually required for the production of these reflexes [42]. Therefore LA function does not yet seem well defined.

BC and LA muscles are innervated by motoneurons located in the spinal nucleus of the *bulbocavernosus* (SNB) [56]. This neuromuscular system constitutes a sexually dimorphic system. SNB motoneurons are significantly larger and more numerous (about 250) in males than in females (about 60) [4, 22]. The sexual dimorphism of LA muscle is likewise related to, on one hand, the number of myofibers (about 6000 in males vs 500 in females) and, on the other, the myofiber cross-sectional area (about 600 μm^2 in males vs 70 μm^2 in females) [61].

This sexual dimorphism is due to the extreme responsiveness of the neuromuscular system to testosterone, at the level of both muscles [24, 10, 7, 14] and motoneurons [4, 5, 14].

The androgen sensitivity is related to the presence of androgen receptors in both perineal muscles [29, 34] as in motoneurons. Androgen receptors have been identified in neonatal male rat muscle by day 2 after birth [17] but nothing is known about androgen receptors in embryonic LA muscle. Neonatal spinal motoneurons accumulate androgens from the second week after birth [17].

Many studies have suggested that the perineal muscles are the primary target for androgens in the masculinization process of this neuromuscular system [12, 35]. However, sexual differentiation of this neuromuscular system occurs during different hormone-sensitive stages [6] and probably involves a network of interactions between both neuron and muscle levels.

In the present review, with the aim of contributing to knowledge of the masculinization process, we report our results concerning the development of LA muscle in male and female rats. In the first part we describe the temporal pattern of muscle fiber formation during the perinatal period. Comparison between the temporal pattern of myofiber formation and fetal and postnatal testosterone secretion in males in addition to testosterone manipulation of newborn females led us to make assumptions about the mechanisms for establishment of sexual dimorphism.

In the second part, we describe the temporal pattern of myofiber enlargement and we show how the rise in plasma testosterone levels during puberty amplifies sexual differentiation of the LA muscle. Testosterone treatment of male and female rats before the normal age of puberty as well as of 2-month-old females confirms that alterations observed during pubertal growth, in males, are controlled by testosterone.

MUSCLE FIBER FORMATION IN LA MUSCLE OF MALE AND FEMALE RATS

Prenatal development

1) Ultrastructural study of the LA muscle in E18-E22 fetuses

LA muscle development is similar to the development of other non hormone-dependent skeletal muscles such as intercostal muscles [32], *extensor digitorum longus* muscles [46, 47] or lumbrical muscles [51]. Therefore, in the present study we focused on the temporal pattern of development.

Pregnancies were dated by the detection of spermatozoa in the vagina, that day being designated Eo.

At E18, the LA muscle consisted of multinucleate primary myotubes and a population of pleomorphic mononucleated cells (e.g. myoblasts and other mesenchymal cells, such as those destined to form muscle connective tissue). The myotubes were interpreted as the primary generation of myotubes owing to their morphological aspect: they were large myotubes with relatively pale cytoplasm as compared to the mononucleate cell population. They contained bundles of myofilaments and central euchromatic nuclei (Fig. 1; a, b).

At E19-E20, a new generation of myotubes was present in close association with primary myotubes. These myotubes were small and had a dark-stained cytoplasm containing myofilaments and heterochromatic nuclei. They are interpreted as secondary myotubes (Fig. 2; a). It has been shown by other investigators that the formation of secondary myotubes results from the fusion of myoblasts. Primary myotubes act as a "cellular skeleton" to guide the longitudinal growth of the new myotubes [31, 15].

During days E20 to E22, the population of secondary myotubes was characterized by its heterogeneity based on diameters and nuclei-cytoplasmic staining intensity. Myotubes were enclosed in "clusters" surrounding by a well defined basal lamina. Clusters, as already described by other authors [32, 46, 47], consist of one large diameter mature primary myotube, one to three small diameter less mature secondary myotubes and occasionally a mononucleated cell (Fig. 1; c and Fig. 2; a).

Usually secondary myotubes were easily recognized by the fact that they insert into primary myotubes by a series of complex interdigitating folds. However, once they have achieved maturation, they separate from the cluster perhaps accompanied by a younger myotube or even a mononucleated cell. This observation suggests that these mature myotubes may serve as "primary" myotubes for new clusters, acting as scaffolds for the formation of other generations of myotubes. Thus it becomes very difficult to distinguish these mature secondary myotubes from primary myotubes.

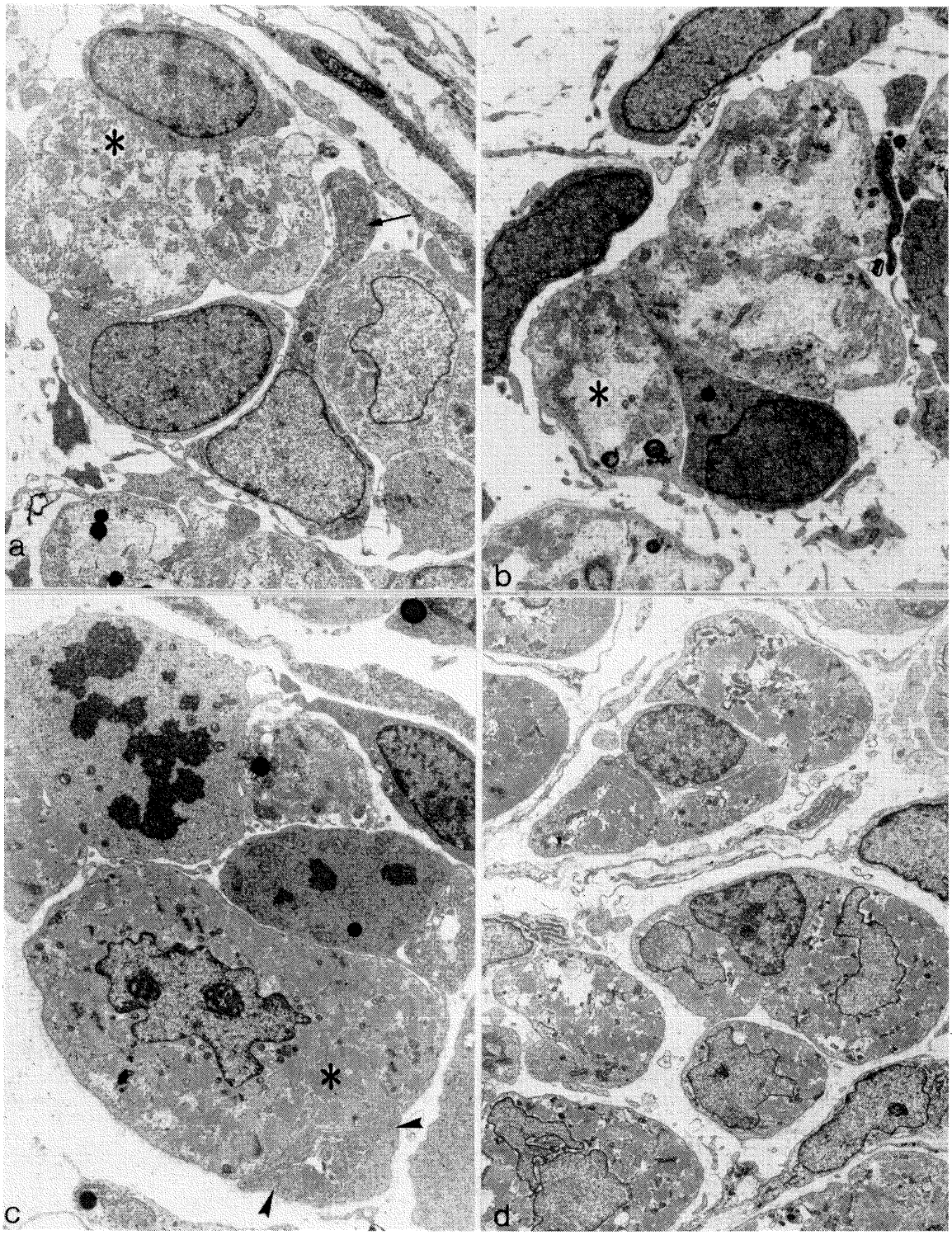

Figure 1. Electron micrographs of male (a, c) and female (b, d) LA muscles.
(a) E18: four primary myotubes with pale cytoplasm containing bundles of myofilaments (*). A mononucleate cell extends a process (↑) between two myotubes. x4000
(b) E19: a dark stained mononucleate cell (●) is insinuated into the interspace between adjacent primary myotubes (*). x3700
(c). D2: a large mature primary myotube (*), associated with a small secondary myotube (▲) and a mononucleate cell (▲), forming a cluster. Two mononucleate cells are dividing. x9000

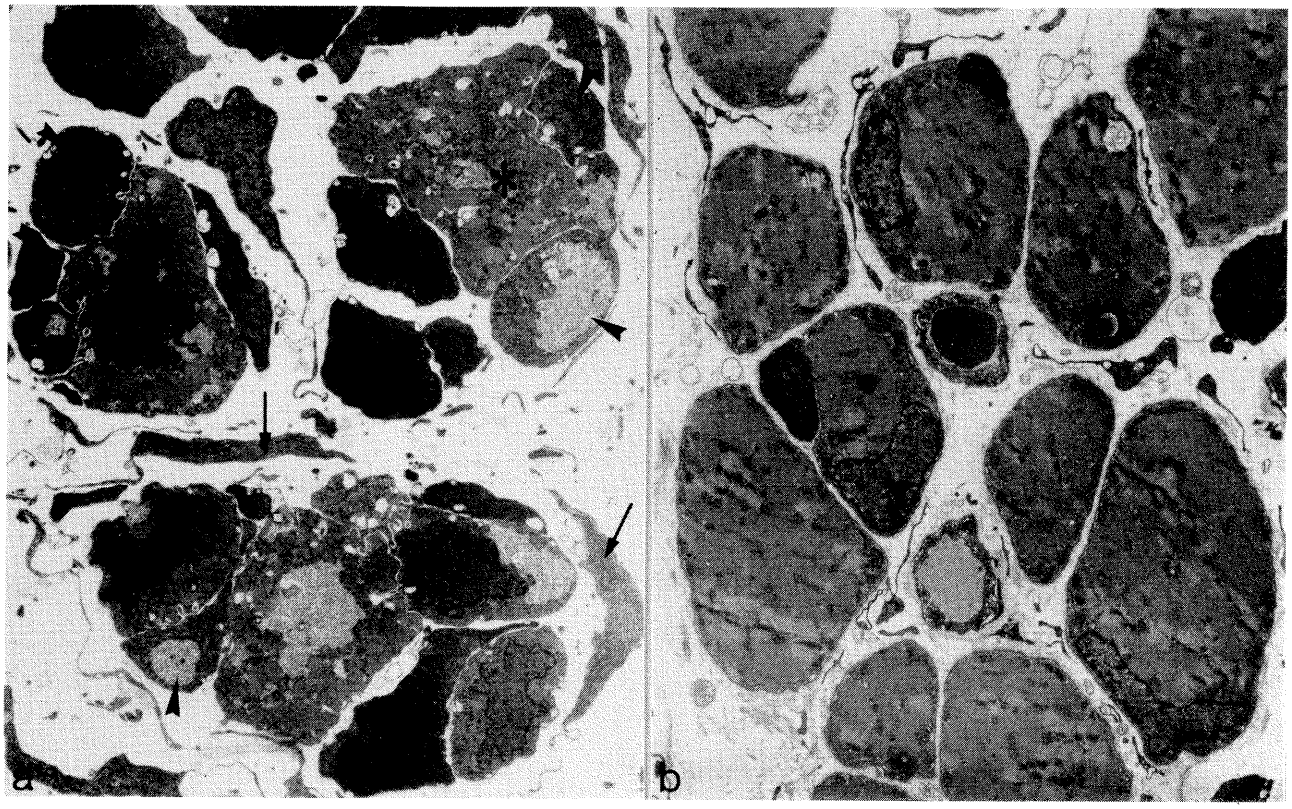

Figure 2. Electron micrographs of male LA muscles.
(a) E20: three clusters consisting of a large primary myotube (*) and small myotubes, interpreted as new generations of myotubes (▲). Cell processes surrounding clusters are part of fibroblasts (↑). x3500
(b) D11: muscle consists almost of mature myofibers characterized by peripheral nuclei and cytoplasm packed with myofibers. x3700

2) Quantification of the myotube number in E18-E20 fetuses

Myotubes were counted in entire cross-sections of male and female LA muscles at E18, E19 and E20 (Table 1).

At E18, when only primary myotubes were present, the number of myotubes was the same in both sexes (male 135 ± 9 vs female 136 ± 9).

At E19, when secondary myotubes begin to form, we observed a slight difference between sexes: the myotube number was 204 ± 10 in the male muscle while in females it remained at the E18 value. Between E19-E20, the myotube number increase continued in males (369 ± 47); a slight increase was also observed in females at E20 (194 ± 3).

Table 1. Quantitative analysis of muscle cells from E18 to adulthood in male and female muscles

	MALE	FEMALE	
E18	135 ± 9	136 ± 9	MYOTUBE NUMBER
E19	204 ± 10	127 ± 12	
E20	369 ± 47	194 ± 3	
D0	680 ± 190.8	234 ± 71.3	MUSCLE UNIT NUMBER
D5	2726 ± 298	355 ± 159	
D12	5895	953	MUSCLE FIBER NUMBER
ADULT	5300 ± 687*	473 ± 251	

*Value obtained from Galavazi and Szirmaï [19]

The ultrastructure observation at E19 of a new population of myotubes, i.e. the secondary myotubes, is correlated with the increase in total myotube number at least in the male.

Therefore, counts of myotubes show that sexual dimorphism appears at E19 and seems to be related to the occurrence of secondary myotubes. Because myotube addition was sexually dimorphic, it was thought to be under the control of sex hormones. Testosterone production in male rats starts at E15-E16 [25] and the plasma testosterone level peaks at E18-E19 [67]. Hence, secondary myotubes occurring on days 19-20 are formed by fusion of myoblasts already present when the plasma testosterone level rises. Therefore different events such as myoblast proliferation or fusion may be enhanced by the male hormone. This assumption is in accordance with previous studies which report that the formation of secondary myotubes is sensitive to environmental variables such as innervation [52] and maternal nutrition [68], while, in contrast, the number of primary myotubes appears to be fixed and insensitive to experimental manipulations [52]. However, plasma testosterone levels do not stay high more than 2 days and return to values not consistently higher than those of females [67]. Is this imprinting sufficient to explain the dramatic increase in myotubes which subsequently form the bulk of fibers in adult muscles? Myoblasts present at the time when plasma testosterone peaks may constitute a pool of testosterone-imprinted myoblasts and may retain the capacity to divide for several days. Testosterone would therefore be a participant in the maintenance of myoblasts in the cell cycle.

Postnatal development

1) Ultrastructural study of LA muscle

The LA muscle development was studied in both sexes from the day of birth (D0) until D11.

At birth, as in the last days of the prenatal period, the developing muscle contains a large population of mononucleated cells, with clusters and mature independent myotubes, the last two surrounded by a well-defined basal lamina.

The period between D0 and D3-D4 is marked by the presence of immature myotubes and replicating cells within the clusters. However, these young myotubes and cells are more frequently encountered in male muscle (Fig. 1; c). In contrast, in female muscle clusters, most of the secondary myotubes are mature and very few dividing cells are present along the myotube walls (Fig. 1; d).

The studied period was also characterized by the increase in the number of mature independent myotubes which later differentiate to myofibers with peripheral nuclei and cytoplasm packed with myofibrils.

At D5, in both sexes, LA muscle consists of clusters containing 2 to 3 myotubes, independent myotubes and myofibers.

At Day 11, the muscle consists almost entirely of mature myofibers. Fibroblasts associated with scant collagen loosely subdivide myofiber groups into rudimentary muscle bundles (Fig. 2; b).

2) Quantitative study

LA muscle was examined with the light microscope because it is well-suited to counting all muscle cells during postnatal development for quantitative analysis [62]. We used the term "muscle units" (MU) to define the cellular components of the muscle, i.e.: clusters, mononucleate cells (except typical fibroblasts), independent myotubes and muscle fibers. The number of MU per muscle was counted on entire transverse sections of LA muscle (Table 1).

At D0, female LA muscles already contained fewer MU (234 ± 71.3) than did males (680 ± 190.8). The number of MU in females increased by about 52% between D0 and D5, whereas the average MU number increased by about 300% in the male over the same period. In both sexes, about 40% of the MU counted on D5 were independent myotubes or mature myofibers, whereas 60% were still clusters. It will be noted that the number of MU at D5 (355 ± 159) in the female muscle was not much lower than the number of fibers in the adult (473 ± 251), while in contrast, the number of MU at D5 in the male muscle (2726 ± 298) was far less than the number of fibers counted in muscle of 11-day-old male (5900). These results show that muscle fiber formation is not complete at D5.

Between D5 and D11, the process of increase in independent myotubes (i.e. future myofibers) is of less magnitude in females than in males, this is due to the lower number of clusters in female muscles and to the weak formation of the secondary myotubes in these clusters. At D11, all MU consisted of mature myofibers.

To complete "the fiber number life story " it will be noted that value obtained from muscle of 11-day-old male is quite similar to those reported by Galavazi and Szirmai [19], 5300 ± 687 for male muscles (Table 1).

Muscle development appears to be a continuous process, not altered by birth. However, morphological observations give evidence of an intense proliferative activity of myoblasts. Moreover, MU quantification shows that myofiber addition essentially occurs during the postnatal period. Although the establishment of sexual dimorphism is obviously correlated with the rise in plasma testosterone level at E18-E19, the question arises of whether testosterone could also act on myofiber formation during the first postnatal days. Indeed the fetal testosterone peak is followed by a high but shorter increase in serum testosterone level at birth, between 0-2 hours [11]. It is well known that this transient peak in serum testosterone has powerful effects on the process of masculinization of the central nervous system of the rat [11]. Moreover, it has been shown that inhibition of neonatal secretion results in a definitive reduction in the weight of accessory sex organs such as epididymis, vas deferens and seminal vesicles [27]. These results suggest that the neonatal testosterone surge in addition to the fetal peak, could contribute to the masculinization of the secondary myotube population.

Effects of neonatal hormonal manipulation on female LA muscles

Testosterone treatment of newborn females evaluated 2 months later induces an increase in the number of muscle fibers only if testosterone is given before D6 (Table 2). Testosterone given on the day of birth (D0) has a significantly greater effect than testosterone given on D1-D5. None of the muscles of the treated animals responded to testosterone after D5 (Table 2). The increase in fiber number induced by testosterone injection is a permanent effect as shown by muscle fiber counts in LA muscles of 15-month-old females [62].

These results confirm and extend those of other investigators [2, 13]. Moreover, they strengthened the assumption that the neonatal testosterone peak interferes in the myofiber addition process, since our results demonstrate that androgen-sensitive myoblasts are still available for stimulation by exogenous androgen, at least during the first postnatal days.

MUSCLE FIBER ENLARGEMENT IN LA MUSCLE OF MALE AND FEMALE RATS

Muscle fiber growth before puberty

From D15 to D20, general fine structure of both male and female LA muscle is quite similar. Muscle fibers appear round-shaped, separated by loose connective tissue. Myofibrils are packed together and exhibit a very clear cross-striation. Nuclei are located at the periphery of fibers, satellite cells [39] are occasionally seen between sarcolemma and basal lamina (Fig. 3; a, b). In mature myofibers, myonuclei are postmitotic and satellite cells represent the only source of new myoblasts which can undergo mitosis after several stimulations [43].

The measurement of the fiber cross-sectional area (csa) (Fig. 4) shows no significant difference on day 15 between either sexes (male 34.31 ± 12.77 μm^2 vs female 39.49 ± 11.57 μm^2). On D20 the average csa appears slightly larger in male muscle (male 76.13 ± 15.36 μm^2 vs female 50.45 ± 10.68 μm^2). Between D20 and D30, increase in the average csa is more marked in male than in female muscle (respectively 124.4 ± 31.86 μm^2, 57.7 ± 16.62 μm^2). Quantification of both myonuclei and satellite cell nuclei (numbers are respectively expressed as the ratios of the number of myonuclei and satellite cell nuclei to the number of fibers x100) at electron microscope level shows no significant difference between the male and female LA muscle. Therefore on D30, before the onset of puberty, LA muscle shows a weak sexual dimorphism related to the cross-sectional area which is two-fold greater in the male (Fig. 4).

Table 2. Effect of TP on the number of female LA muscle fibers.

DAY OF INJECTION	NUMBER OF FIBERS
D0 (day of birth)	2454 ± 323
D2	1546 ± 30
D5	1243 ± 330
D6	611 ± 114
D9	508 ± 232

Muscle fiber growth during puberty

LA muscle growth was examined in early puberty (D42), puberty (D51) and in adulthood (D78) in male and female rats of the same age by morphological and quantitative studies. The most striking observation at electron microscope level was that the shape of the fibers changes from round to polygonal in males with increasing age (Fig. 2; b and Fig. 3; a). The space between the fibers is reduced. These morphological changes are less pronounced in female LA muscle. The sarcolemma is closely apposed to the peripheral myofibrils in male, while in female LA muscle, the sarcolemma shows evident undulations (Fig. 3; b).

Between D30 and D42, muscle fiber enlargement is enhanced in the male, while in the female no change is observed. From D51 to D78, a sharp increase is observed in the male leading to a fiber csa about 10-fold higher in males than in females (male 718.67 ± 147.76 μm^2 vs female 65.88 ± 21.73 μm^2). Hence, in the male, LA muscle undergoes considerable hypertrophy whereas in the female, LA muscle fails to grow (Fig. 3; a, b and Fig. 4). This muscle hypertrophy can be related to the rise in circulating testosterone which occurs around D40 [18] in the male, while in the female, testosterone level remains very low.

The effects of testosterone on muscle growth regulation, i. e hypertrophy or atrophy, are usually considered to be anabolic. Our quantification of the number of myonuclei (MN) and satellite cell nuclei (SC) demonstrates that the anabolic effect of endogenous testosterone is accompanied by a mitogenic effect on the satellite cell population. On D30, in male and female LA muscle, satellite cells are quiescent. Satellite cell nuclei and myonuclei number are similar in both sexes (male SC: 5.97 ± 0.72, MN: 59.14 ± 3.65 vs female SC: 6.39 ± 1.52, MN: 52.26 ± 6.53) and do not increase until D42. Just after the onset of puberty (between D42 and D47), a transient increase in the satellite cell nuclei number is observed only in male muscle. This satellite cell proliferation is followed by a subsequent increase in myonuclei number from D47 to D78. Hence, during this period, the myonuclei number has increased by about 50% in male LA muscle, whereas the myonuclei number remains unchanged in the female (Joubert et al., in press). The lag time observed between the increase in the numbers of satellite cell nuclei and myonuclei in the male, as well as the well-known postmitotic nature of the myonuclei, indicate that new myonuclei originate from the fusion of the satellite cells with myofibers. These results suggest that satellite cell proliferation is induced by the rise in plasma testosterone level.

In summary, during puberty, development of LA muscle is characterized by an increase in muscle fiber enlargement

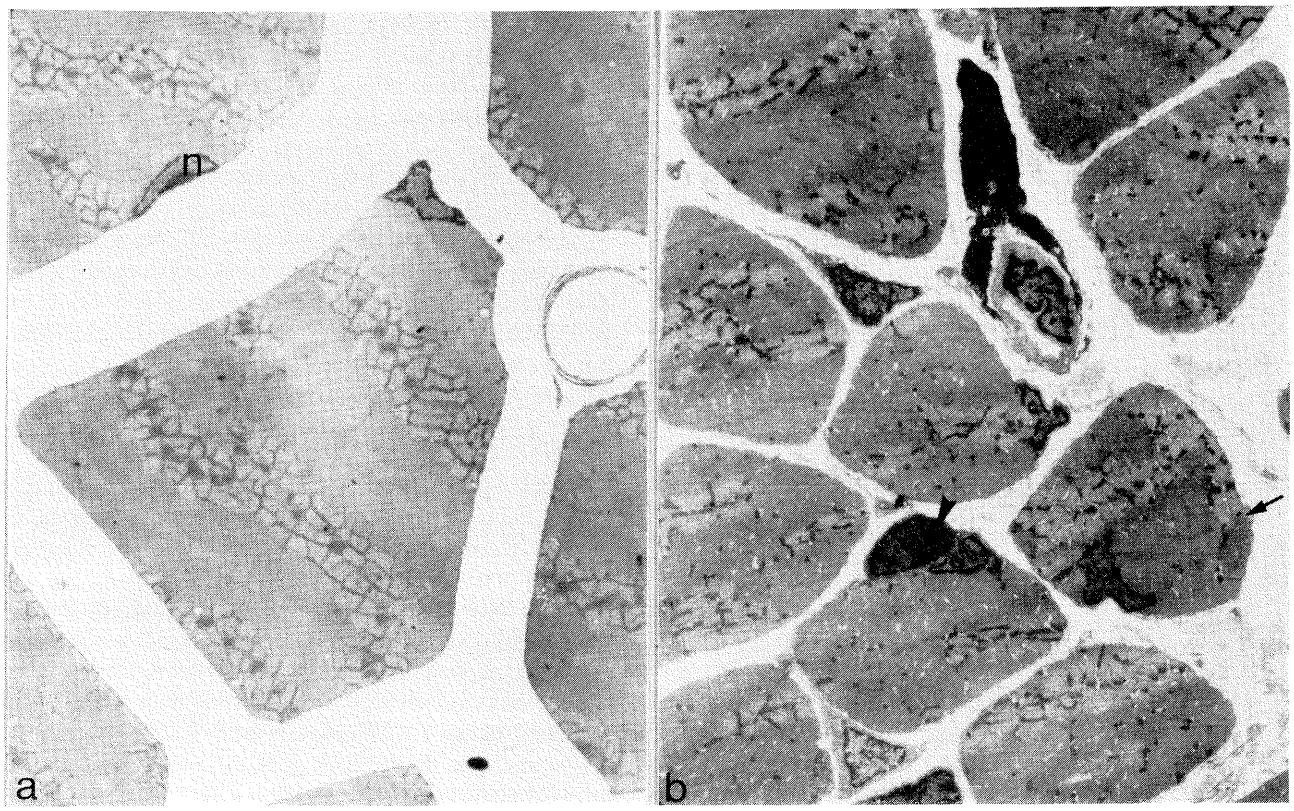

Figure 3: Electron micrographs of portion of transverse sections of 78-day-old male (a) and 78-day-old female (b) LA muscles. Same magnification. Note the striking difference in the csa of the muscle fibers. In males, fibers usually are polygonal shaped, nuclei (n) are located at periphery of the fibers. In females, fibers are round shaped, nuclei are indented and sarcolemma shows undulations (↑); satellite cells (▲) are occasionally seen between sarcolemma and lamina. x3000.

in the male with no corresponding muscle fiber enlargement in the female. Such hypertrophy induced by testosterone treatment has already been described by several authors in castrated male rats. These authors reported an increase in fiber diameter and myonuclei number [19], as well as in DNA synthesis [50]. However, they did not discuss the origin of the new myonuclei. Our investigations strongly suggest that during puberty endogenous testosterone regulates muscle hypertrophy and plays a hitherto unrecognized role in satellite cell proliferation.

Effects of hormonal manipulation

1) Influence of exogenous testosterone before the onset of puberty

30-day-old male and female rats were given testosterone before the normal age of puberty, which occurs in the male around D40 [18]. This treatment generates muscle fiber enlargement, transient satellite cell proliferation, and myonuclei increase not only in male but also in female muscle. It is noteworthy that, on one hand, these induced processes take place at the same rate in both sexes and, on the other, they start earlier (around D33) than observed in the male during "physiological" puberty. On D78, myonuclei number is the same in both male (97.92 ± 6.88) and female (94.8 ± 7.54) LA muscle (Joubert et al., in press). These results confirm those obtained from the study of muscle during puberty; testosterone actually controls the myonuclei number *via* satellite cell proliferation and fusion processes.

2) Influence of exogenous testosterone in adult females

60-day-old females were given testosterone treatment. The sequence of events observed in the muscle fibers (i.e. enlargement of fiber csa, transient satellite cell proliferation, myonuclei number increase) was the same that observed in 30-day-old treated females. In untreated 60-day-old females, the average csa was 72.7 ± 8.6 μm^2, while in treated 90-day-old females it reached a maximum of nearly six times this figure (427.3 ± 21.3 μm^2). Myonuclei number increased from 57.23 ± 5.59 at the time of injection (D60) to 103.21 ± 16.32 one month later [28].

In contrast, during the same period, the number of fibers does not vary significantly, indicating that testosterone does not affect the number of fibers in female adult LA muscle.

These hormonal manipulations demonstrate that there is no strict temporal constraint on masculinization of myofiber diameter and myonuclei number in contrast to masculinization of fiber number. Moreover, we can conclude that only the low circulating testosterone level in the

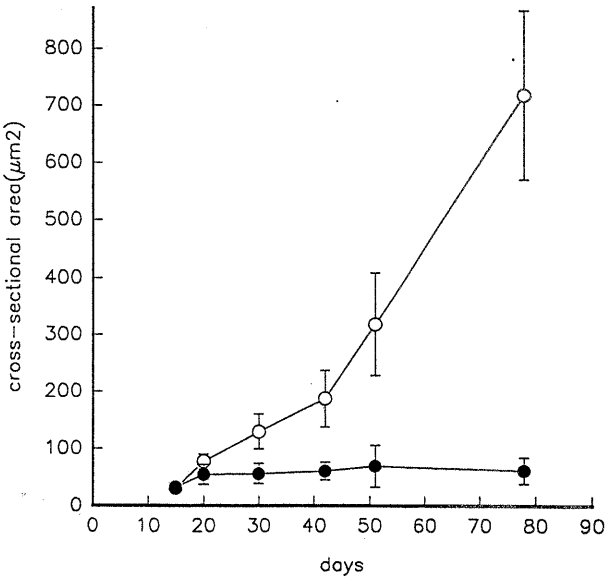

Figure 4. Average cross-sectional area(csa) of muscle fibers in the LA muscles of male (o) and female (●) rats aged 15-78 days. Each point represents the mean ± SD of two muscles (150 muscle fibers per *muscle were measured). On day 30, csa is already sexually dimorphic. In the female, fiber csa remains constant from day 20 to day 78. In the male, fiber csa increases sharply from day 51 to day 78 leading to an accentuated sexual dimorphism.*

female during puberty and in adulthood prevents the LA muscle acquiring male characteristics, i.e. muscle fiber enlargement and myonuclei number increase.

Discussion

Altogether, our results demonstrate that testosterone acts on development and growth of the LA muscle during two critical periods: the perinatal period and puberty.

It is well-known that gonadal steroids have two influences on developmental events, namely "organizational" and "activational" [49]. The former effects are structural and permanent, whereas the latter effects are altered or lost in the absence of the relevant hormone. As has been shown for the nervous system [40, 21] and particularly for the SNB [7, 2], the organizing influence of androgens in developing muscle ends on postnatal D5. Beyond D5, testosterone no longer stimulates myofiber formation in females. However, this does not mean that myofiber addition is complete on D5 in the male LA muscle, since we have observed that myofiber number in 11-day-old male is much greater than in 6-day-old males. This further addition is probably due to the completion of a process initiated before D6. Thus, on the basis of our results we propose that testosterone controls in a permanent manner the number of fibers in adulthood by acting on the secondary myotube formation during a "window of time" extending from E19 to D5.

The activational effects of testosterone are almost all observed during puberty. Indeed, we have shown that the rise in endogenous testosterone enhances enlargement of fibers and induces a transient satellite cell proliferation that leads to a permanent myonuclei increase. Thus, in addition to anabolic action on protein synthesis [9, 8], testosterone promotes muscle cell proliferation even outside the perinatal period. The hypertrophy of the muscle fibers is not definitive since castration provokes muscle atrophy associated with a decrease in the muscle fiber diameter without fiber loss [63]. However, DNA decreased after castration [50] but no study has examined whether this decrease is due to a loss of myonuclei.

The activational influences of testosterone on LA muscle appear to be exerted throughout life since hormonal treatment induces activational effects both earlier than the pubertal period and later in adult females. Moreover, the fact that these effects can be obtained in young or adult females demonstrates that perinatal testosterone imprinting is not necessary to make the muscle hormone sensitive.

Our results concerning perinatal and pubertal growth of the LA muscle raise the question of the site of testosterone action: in the neuromuscular system composed of perineal muscles and SNB, which is the primary target of testosterone? Many studies have demonstrated that androgens regulate the development of the SNB [47, 1, 3] and have important effects on the morphology of the SNB motoneurons in adulthood [20, 1, 54].

Further support for the role of androgens in SNB masculinization is confirmed by the markedly feminine appearance of the SNB in male testicular feminized (tfm) rat mutants or in males treated with flutamide during prenatal period [6, 22]. The control of androgens upon SNB size is also evidenciated by the increased number of SNB neurons in adult females when they are given testosterone in perinatal period [58]. A similar testosterone treatment of newborns females equally induces an increase in the number of muscle fibers [62].

Motoneurons undergo their final mitosis on E14. At E18, there are no sex differences in the number of SNB motoneurons. Sexual dimorphism related to their number is established by differential cell death of neurons from the day before birth through postnatal D10 [45]. We have shown in this study that sexual dimorphism related to myotube formation starts at E19. Therefore the establishment of muscle sexual dimorphism takes place 2 days before the occurrence of motoneurons death. This observation favours the hypothesis that muscle cells are the primary testosterone target. In this case, testosterone regulates the rate of motoneurons death *via* its control of muscle size, i.e. myotube number. The period during which motoneuron death occurs is well-correlated with the period of myofiber formation since both of them end around D10-D11.

The myogenic origin of the hormone sensitivity within this neuromuscular system is supported by many studies (for review see 16, 35, 3, 12). Although secondary myotubes seem to require direct hormonal stimulation for the initiation of their development is not at variance with the finding that, finally, fiber addition is regulated by the interaction of nerve and hormone [52, 60].

Although muscle cells may be considered as the primary target of testosterone, the question of the precise site of hormonal action is not resolved. Myotube formation is closely dependent on the presence of myoblasts: Ross and al. [52] have demonstrated a very high correlation between the number of mononucleate cells (including myoblasts) and the rate of secondary myotube formation in rat lumbrical muscle. It is then very likely that one of the ways androgens can affect myotube formation is by stimulating myoblast proliferation. One must find which cell population within the muscle is responding to androgens in order to know how testosterone stimulates myoblast mitosis. Indeed, testosterone can enhance myoblast proliferation either by a direct action on the muscle cell precursors or by an indirect route, acting on mesenchymal cells, for instance, which could subsequently produce myoblast proliferative factors. Likewise, during puberty the mitogenic effect induced by testosterone could be exerted directly on satellite cells themselves, or indirectly *via* a primary action on other targets, such as myonuclei or innervating SNB motoneurons. This issue could be resolved by studying androgen receptor expression in the developing LA muscle, using immunostaining and *in-situ* hybridization techniques.

Address correspondence to:

Christine Tobin, Université P&M Curie, Institut des Neurosciences, Département de neurobiologie des signaux intercellulaires, 7, quai St Bernard, bat. A 3ème étage, F-75252 Paris Cedex 05, Tel (33) 1 44273143, Fax (33) 1 44071585.

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