Postnatal cytochemical development of muscle fibers in segmental tail muscles of the rat

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SUMMARY

Postnatal development of extrafusal and intrafusal muscle fibers was examined histochemically in segmental tail muscles of the rat. At birth all fibers show a strong reaction for myosin ATPase, uniformity in diameter, and homogeneity in staining intensity. During the first postnatal week, the muscle fibers undergo gradual hypertrophy and hyperplasia but they all maintain the same intense homogeneous staining pattern for the enzyme. By day 9, further differentiation of the muscle fibers results in the formation of a second intrafusal fiber type while the extrafusal fibers are still relatively homogeneous. Finally, two kinds of extrafusal fiber and a third type of intrafusal fiber can be distinguished by day 21. This histochemical fiber pattern is essentially maintained in the adult.

These findings show that fiber type development in rat tail muscles lags behind the usual time course of myogenesis known to occur in more rostral regions of the animal. It also indicates that histochemical differentiation of intrafusal fibers in these muscles does not parallel that which occurs in extrafusal fibers. It is likely that arrival and initial contact of sensory nerve terminals on developing intrafusal fibers at day 7 directly influences their relatively early histochemical heterogeneity.

INTRODUCTION

There is little doubt that during development, mammalian skeletal muscles undergo considerable changes in their biochemical and physiological properties and in their fiber type pattern, particularly during the late prenatal and early postnatal stages (Close, 1972). Those changes, however, are known to differ according to the animal species and the particular kind of muscle examined. Cephalic muscles in the rat mature earlier than caudal muscles (Boethius, 1969). Moreover, rat intercostal muscles become fairly well differentiated in the prenatal period (Kelly & Zachs, 1969), while certain hindlimb muscles may not complete their development until one or two weeks postnatally (Dubowitz, 1965).

A variety of histochemical techniques have been used to elucidate biochemical and physiological parameters occurring at the muscle fiber level especially

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during development (Davies, 1972). Most studies of myogenesis utilizing these techniques, however, have either been carried out on mammalian limb muscles (Close, 1972) or on muscles located in more rostral regions of animals.

The small segmental intertransverse tail muscles of the rat represent the most caudal muscles in this species (see Schumacher, 1909), and they are known to possess unique properties which set them apart from muscles in more rostral parts of the animal (Thompson, 1970). Their muscle spindles are notably different from those found in other mammalian muscles. They are relatively simple with respect to their innervation and in some ways they are thought to resemble spindles of more primitive vertebrates (Steg, 1964; Thompson, 1970).

These small muscles span the caudal vertebrate of the rat tail and are able to perform fine, delicate movements. They function in controlling the position of the tail (Andrew, Leslie & Thompson, 1973). While their physiological and contractile properties have been studied in detail (Steg, 1964; Andrew & Part, 1972, 1974), little is known about the fiber types comprising these muscles and nothing is known about their ontogenetic development.

This report deals with the postnatal development of extrafusal and intrafusal fibers in the lateral intertransverse tail muscles of the rat. In this study we have primarily chosen to use the myosin ATPase histochemical technique since it can be directly correlated with speeds of contraction in muscle (Barany, 1967) and because it reveals subtle cytochemical differences between the muscle fibers (Ovalle & Smith, 1972). While some preliminary ultrastructural findings are also presented in correlation with the histochemistry, a more detailed electron microscope examination of these muscles during development will be published elsewhere.

**MATERIALS AND METHODS**

Neonatal and early postnatal rats of the Sprague Dawley strain, varying in age from day 0 to day 21, were used in this study. Animals were selected from four separate litters and killed with ether.

For histochemistry, a cross-sectional segment from the proximal portion of each rat's tail was cut and removed with a razor blade, dipped in talcum powder, and rapidly quenched in 2-methylbutane cooled to -70 °C with dry ice. Each tail segment was then transferred to a cryostat (-20 °C) and 10 μm serial frozen sections were cut in the transverse plane. Myosin ATPase activity in the proximal tail muscles was demonstrated by the Guth & Samaha (1970) modification of the Padykula & Herman (1955) procedure. Best results were obtained by alkaline preincubation at pH 10.4 for 15 min and incubation in the substrate at pH 9.4 for 90 mins at 37 °C. Control slides were processed simultaneously using a specific inhibitor for myosin ATPase (PHMB); absence of staining under these control conditions indicated that the reaction, as
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normally carried out, did demonstrate myosin ATPase (Ovalle & Smith, 1972). The lateral tail muscles of animals from the following developmental stages were examined: day 0 (newborn), 1, 3, 5, 7, 9, 10, 14 and 21.

For light and electron microscopy, similar proximal tail segments were removed in toto from each animal. The overlying skin was removed, and each segment was immersed in Karnovsky’s (1965) fixative diluted to 50% with 0.1 M cacodylate buffer at pH 7.3 for several hours. Segments were then washed in repeated changes of buffer, postfixed in 1% aqueous osmium tetroxide for 1 h, stained in a saturated solution of uranyl acetate, dehydrated in a graded series of ethanols, and embedded in a 1:1 mixture of Epon/Araldite. To localize the lateral pair of tail muscles, transverse sections (1–2 μm thick) of each segment were cut, stained with 1% aqueous toluidine blue, and examined with the light microscope. Once proper identification was made, each block was trimmed down to the desired area. Thin transverse sections were subsequently cut with a diamond knife, mounted on coated copper grids, stained with lead citrate (Reynolds, 1963) and examined in a Phillips 200 electron microscope.

Diameters of muscle fibers at various stages of development were measured with a calibrated ocular micrometer fitted to a Leitz optical microscope. Frozen cross-sections of the appropriate tail muscles stained for myosin ATPase were examined, and the mean diameter of two measurements taken at right angles to each other was calculated for each fiber.

RESULTS

Light microscopy

A detailed description of the gross anatomy of the rat tail and its musculature has been provided by Thompson (1970). The tail contains three bilateral pairs of segmental intertransverse muscles which span each vertebra. A transverse section of a neonatal rat tail (Fig. 1) shows that each muscle pair is located on either side of a vertebra. Two dorsal, two ventral, and two lateral muscles are easily distinguished during the early stages of postnatal development. Only the lateral pair of muscles in the proximal part of the tail (segments 1–10) were examined in this study.

Histochemistry

At birth, all the muscle fibers comprising the lateral tail muscles stain darkly for myosin ATPase and are homogeneous in their staining intensity for this enzyme (Fig. 2). Similarly, fibers in the ventral and dorsal muscles were found to exhibit the same homogeneous staining pattern. At this stage the muscle fibers are quite small and uniform in their cross-sectional diameters. They contain centrally located nuclei which appear as clear spaces in the centers of most fibers.
Fig. 1. Transverse section of a neonatal rat tail. The large tail artery (a) marks the ventral side. Three bilateral pairs of muscles surround the developing vertebra in the center. Two ventral and dorsal muscles (arrowheads) and the two lateral muscles (L) are indicated. Toluidine blue stain. × 45.

Fig. 2. Newborn rat tail stained for myosin ATPase. All fibers in the ventral (V), dorsal (D), and lateral (L) tail muscles react strongly for this enzyme. The developing vertebral bone (B) is indicated. × 100.
At day 1, all fibers in the lateral tail muscle maintain the same uniform staining intensity seen at birth (Fig. 3). At this stage, intrafusal fibers of muscle spindles are clearly distinguished from the surrounding extrafusal fibers on the basis of their cross-sectional diameters. The intrafusal fiber diameter range is 1.5–2.0 \( \mu \text{m} \) \((n = 22)\) while that for the extrafusal fibers is 4–10 \( \mu \text{m} \) \((n = 50)\). At this stage, muscle spindles usually contain two small intrafusal fibers which appear homogeneous in their staining reaction and uniform in their cross-sectional diameters. Parallel spindles similar to those described by Thompson (1970) were commonly encountered, despite the fact that spindle capsules are not easily seen at this stage.

During the first week of postnatal development, the intrafusal and extrafusal fibers maintain their intense and homogeneous staining pattern for myosin ATPase. In this period, however, all muscle fibers show a gradual increase in their number and in their cross-sectional diameters.

By the ninth day of postnatal life, the muscle fibers first begin to show differences in their staining reaction for myosin ATPase (Fig. 4). At this time the intrafusal fibers have now doubled in number (usually four per muscle spindle) and have reached a diameter range of 4–6 \( \mu \text{m} \) \((n = 18)\). They can now be divided into two distinct populations: dark-staining fibers and less dense (intermediate) staining fibers. The extrafusal fibers, which now measure 7–20 \( \mu \text{m} \) in diameter \((n = 50)\), do not show such clear-cut differences in their staining intensity (Fig. 4).

By day 21, the muscle fiber type pattern demonstrated with the myosin ATPase reaction is similar to that seen in tail muscles of the adult rat (unpublished observations). At this time, two types of extrafusal fiber and three kinds of intrafusal fiber are distinguished (Fig. 5). The majority of extrafusal fibers (72%) stain darkly for myosin ATPase and exhibit large cross-sectional diameters (25–40 \( \mu \text{m} \)), while the remaining population of extrafusal fibers (28%) are lighter in their staining intensity and have smaller diameters (15–30 \( \mu \text{m} \)). At this stage (day 21) no significant differences in size are noted between the intrafusal fibers examined. Furthermore, their diameter range did not appear to change from that observed at day 9 (i.e. 4–6 \( \mu \text{m} \)). Their staining pattern for myosin ATPase, however, sets them apart from the extrafusal fibers and they can now be divided into three kinds (Fig. 5). One type maintained the intensely dark staining reaction seen previously, the second type was intermediate in its staining intensity, and the third type appeared pale and was very lightly stained.

Most muscle spindles examined at this stage appear similar to those seen in the adult. Most contain four intrafusal fibers, while a few may possess as many as five. The dark-staining fibers are the most prevalent, usually numbering two, while the intermediate-staining fibers are the last numerous in a given muscle spindle.
Electron microscopy

It is known that normal differentiation of mammalian muscle fibers is directly dependent on an intact nerve supply (Karpati & Engel, 1967). In contrast to extrafusal fibers, however, intrafusal fiber differentiation depends initially on the arrival of the sensory (afferent) nerve terminal to the muscle spindle (Werner, 1972). In this study we examined muscle spindles during the first 10 days of postnatal life in order to determine at what stage sensory nerve terminals first establish neuromuscular contact with the intrafusal fibers in the tail. It was found that during the early postnatal period (days 0–5), muscle spindles in the tail are completely devoid of sensory nerve terminals. At day 7, however, naked sensory nerve terminals are first seen approaching and making intimate contact with surfaces of developing intrafusal fibers (Fig. 6). This usually occurred at a time when myofilaments were first beginning to appear in the sarcomplasm of the developing muscle fiber, just before the time (day 9; see Fig. 4) when these fibers could be histochemically divided into more than one type. The sensory nerve terminals at this stage begin to envelop their processes around the intrafusal fibers. They contain scattered mitochondria, neurotubules, microfilaments, and numerous vesicles of the clear and dense-core variety. A 100 Å gap, devoid of any extracellular material, usually separates the growing nerve terminal from the surface of the developing intrafusal muscle fiber (Fig. 6).

DISCUSSION

The results of this study indicate that histochemical differentiation of muscle fibers in rat segmental tail muscles begins after the first week of postnatal life. Differentiation of these muscles, therefore, occurs later than in those muscles located in more rostral regions of animals (Karpati & Engel, 1967; Nystrom, 1968; Davies, 1972). This difference in the time of onset of tail muscle development may be due to the fact that early functioning of these muscles is not as vital to the animal as it is in other muscles, especially those used for locomotion. It has been shown, for example, that functional overload to a muscle can exert a modifying influence on the histochemical differentiation of its muscle fibers (Tomanek, 1975).

Figures 3–5

Fig. 3. One-day-old lateral tail muscle. All extrafusal and intrafusal (arrows) fibers show high myosin ATPase activity. × 900.

Fig. 4. Nine-day-old lateral tail muscle. Two sorts of intrafusal fiber, dark (dark arrows) and intermediate (arrowheads) are seen. The extrafusal fibers remain relatively homogeneous. × 900.

Fig. 5. Day-21 lateral tail muscle. Two kinds of extrafusal fiber (labelled 1 and 2) and three kinds of intrafusal fiber, dark (dark arrows), light (clear arrow), and intermediate (arrowhead), are seen at this stage. × 1350.
Fig. 6. Electron micrograph. Transverse section of a muscle spindle from a 1-week-old animal. Two multinucleated intrafusal fibers are seen closely apposed to each other. Their interdigitating muscle cell membranes (arrowheads) are indicated. One fiber is intimately and partially invested by a developing sensory nerve terminal (S). × 7800.

The uniform dark-staining reaction for myosin ATPase observed during the first postnatal week is similar to that reported in other mammalian neonatal muscles (Guth & Samaha, 1972; Tomanek, 1975). Guth & Samaha (1972) have suggested that this high intensity of myosin ATPase staining at birth does not reflect fast speeds of contraction as it does in the adult. Since mammalian muscles are generally slow-contracting at birth (Close, 1964), Guth and Samaha contend that the degree of myosin ATPase staining in neonatal muscle may not necessarily reveal the actual level of enzymatic activity. They
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suggest that this histochemical staining intensity is due to a close packing of myofibrils and the staining of other forms of ATPase in the neonatal muscle fiber. Despite this disparity between histochemical staining properties and the dynamic nature of the muscle fibers, the fact remains that the cytochemical procedure employed in the present study can be useful for the elucidation of fiber types during myogenesis (Davies, 1972).

The present study shows that intrafusal fiber differentiation slightly precedes that observed in the extrafusal fibers. By day 9, for example, two kinds of intrafusal fiber were clearly demonstrated while the extrafusal fibers were still relatively homogeneous. It may well be that the sensory innervation to the intrafusal fibers sets them apart, developmentally, from the extrafusal fibers (Smith & Ovalle, 1972). The initial formation of neuromuscular contact by growing sensory nerve terminals in muscle spindles at a time (day 7) just prior to the appearance of two histochemically distinct intrafusal fibers (day 9), certainly lends credence to this hypothesis. Ultrastructural studies on developing hindlimb muscle spindles in the rat show that sensory nerves play an important role in the induction of intrafusal fiber differentiation (Landon, 1972; Milburn, 1973; Zelena & Soukup, 1973). The precise nature of their morphogenetic influence, however, is still unknown.

Adult hindlimb muscles, known to have three kinds of extrafusal fiber, possess spindles containing three kinds of intrafusal fiber (Ovalle & Smith, 1972). Tail muscles, however, do not seem to adhere to this extrafusal/intrafusal ratio observed in hindlimb muscles. By day 21, muscle fibers in the tail attain their adult fiber type pattern. At this stage, three kinds of intrafusal fiber and only two kinds of extrafusal fiber were detected. It is possible that the histochemical methods used in this study were not subtle enough to detect three discrete kinds of extrafusal fiber. However, the fact that clear-cut differences could be detected in the three intrafusal fiber types would render this possibility less likely.

Extrafusal motor units in rat tail muscles can be divided into two categories; fast-contracting units and slow-contracting units (Andrew & Part, 1972). It is likely that the two extrafusal fiber types demonstrated histochemically (see Fig. 5) correspond to the two kinds of motor units described by Andrew and Part (1972). The fast-contracting units probably correspond to the dark-staining muscle fibers (labelled 1 in Fig. 5), and the slow-contracting units to the lighter-staining muscle fibers (labelled 2 in Fig. 5).

That the histochemical profile pattern in the intrafusal fibers (three types) does not match that in the extrafusal fibers (two types) might be explained by the fact that rat tail muscles possess unique and unusual functional properties which set them apart from other mammalian muscles (Thompson, 1970). Some of their intrafusal fibers share a common motor (beta) innervation with the extrafusal fibers, a situation which does not normally occur in most other mammalian muscles (Steg, 1964; Andrew & Part, 1974). Beta motor innervation,
on the other hand, commonly occurs in amphibian muscles where extrafusal fibers do not directly match intrafusal fibers either histochemically or ultrastructurally (Smith & Ovalle, 1973). The histochemical disparity observed between extrafusal and intrafusal fibers in rat tail muscles may be due to the presence of this shared motor innervation.

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