Differentiation of fibre types in an extraocular muscle of the rat

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SUMMARY

Mammalian extraocular muscles possess greater variation in structural and physiological properties than any other muscle. The superior rectus muscle of the adult rat contains five morphological fibre types. The differentiation of the muscle into these fibre types in embryonic and postnatal rats were studied by light and electron microscopy, and the distribution of each developing fibre type with its distinctive features was mapped. The muscle of the 18-day embryos did not display the specific structural fibre types that were observed in the adult muscle. Newborn rat muscle exhibited some differentiation that included scattered small-diameter fibres with large myofibrils (fibre type 'B'). As development proceeded, another small-diameter fibre type with small myofibrils (fibre type 'A') appeared in the 6-day postnatal rat muscle. By the end of the first week of development neuromuscular junctions were in evidence in these two fibre types. Postsynaptic folds were rare in the large-fibril fibre, and folds were extensive in the small-fibril fibre. The medium- (fibre type 'C') and large-diameter (fibre type 'D') fibres were fully differentiated with small myofibrils and abundant sarcoplasmic reticulum (SR) by the second week of the development. SR was most abundant in the large-diameter fibre, which constituted the predominant global fibre type in the adult muscle. The postsynaptic folds in the neuromuscular junctions of these two fibre types were highly developed, although the innervation did not extend widely in the global region of the muscle. The last fibre type (fibre type 'E') was fully differentiated with the largest myofibrils, a small amount of SR, and simple neuromuscular junction by the third week of the postnatal development. The superior rectus muscle of the four-week-old rat was differentiated with all fibre types present in the adult muscle. During the third to sixth, and final, week of development, the other types described above exhibited extensive differentiation of characteristic structural features.

INTRODUCTION

It has been shown that extraocular muscles are the most complex muscles in vertebrates and that they exhibit greater variation in structural (Hess, 1961; Peachey, 1966; Kilar斯基 & Bigaj, 1969; Cheng & Breinin, 1966; Miller, 1967; Mayr, 1971; Nag & Peachey, 1972a, b; Alvarado-Mallart, 1972; Harker, 1972; Peachey, Takeichi & Nag, 1974; Davidowitz, Phillips & Breinin, 1977; Nakao & Aoki, 1982) and physiological properties (Hess & Pilar, 1963; Bach-Y-Rita & Ito, 1966; Pilar, 1967; Matyushkin, 1967) than any other muscles (Kelly & Zacks, 1969; Nag, 1972; Nag & Nurrall, 1972; Ontell & Dunn, 1978; Gonyea,

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Although differences in structure and innervation of extraocular muscle fibres were known to light microscopists, there has been no agreement on muscle fibre classification. The recent electron microscope and histochemical studies (Mayr, 1971; Harker, 1972; Nag & Peachey, 1972a, b; Peachey, Takeichi & Nag, 1974) have led to the structural classification of fibre types and innervation in the extraocular muscles of mammals. These studies have shown the presence of five types of fibres in the superior rectus muscles of rats, cats, and sheep.

Since, to our knowledge, there have been no studies on the development or differentiation of fibre types in the extraocular muscle, we have undertaken the present study to examine the differentiation of the fibre types in the superior rectus muscle of the rat during embryonic and postnatal periods. The specific objectives of this study are: (a) to ascertain whether the five fibre types present in the superior rectus muscle of the adult rat undergo differentiation and assume distinctive structural features simultaneously or whether they differentiate sequentially, as the animal develops; and (b) to study the differentiation of neuromuscular junctions, together with their influences on the development of the fibre types.

MATERIALS AND METHODS

Microscopy

Superior rectus muscles of 18-day rat embryos and postnatal rats were examined in this study. In all, fifteen embryos and seven groups of ten postnatal rats of varying ages up to six weeks were used. The head of the embryo was placed in half-strength modified Karnovsky’s fixative (1965) overnight, and then the superior rectus muscle was dissected out and fixed in the half-strength fixative for an additional 4 h at room temperature. Postnatal muscles were fixed in full-strength modified Karnovsky’s fixative, which contained 4% paraformaldehyde and 4% glutaraldehyde. Post-fixation for both embryonic and postnatal muscles was in 1% OsO₄ in 0.15 M sodium cacodylate buffer (pH 7.4) at 0°C for 1–2 h. Dehydration was in a graded series of ethanol followed by propylene oxide. Thick and thin sections were cut, and the thick transverse sections were stained with toluidine blue and photographed. Maps of muscle fibres were prepared. In order to identify the fibres and their exact locations, electron micrographs of fibres were compared with light micrographs from the same region of the muscle at each developmental stage. The thin sections were stained in saturated uranyl acetate in 50% ethanol for 4–6 min and then in lead citrate for 2–4 min and examined with a Philips 200 electron microscope at 60 kV.

Quantitation

The diameter, area, and mitochondria content of the fibre types were measured on the electron micrographs by an instrument called the MOP-3 (Carl Zeiss
Fig. 1. Low-power electron micrograph of a portion of the superior rectus muscle of the 18-day rat embryo, showing the same degree of differentiation of myofibrils (Mb) of the muscle fibres. Note the presence of ribosomes (Rb) around the differentiating myofibrils.
Fig. 2. High-power electron micrograph of two 'B' fibres each with a different degree of differentiation in a superior rectus of the 3-day-old rat. Note the large myofibrils (Mb) with scanty sarcoplasmic reticulum (Sr). The lower fibre is almost completely differentiated, whereas, the upper fibre is in the way of differentiation, showing the assembly of large continuous myofibrils (Mb). M, mitochondria.
Fig. 3. A myotube of a 3-day-old superior rectus, showing abundant myofilaments (Mf) in the sarcoplasm. Note differentiating Z-lines (arrows) and rough endoplasmic reticulum (Rer) with dense material; Ds, desmosome; Ps, polysomes.
Inc.), which was equipped with the modular system for quantitative digital image analysis. The electron micrographs were placed upon the instrument's special tablet, which generated dynamic magnetic currents that were sensed by a stylus at selected contours of the cells and organelles. At intervals of less than 0.1 mm, signals were received by the microprocessor and translated into geometric data. Coordinate points were updated at a rate of 100 mm/sec. The details were stored and statistically analysed by the MOP-3. For the measurement of the diameters and the estimation of the mitochondrial volume in the fibre types, 50 to 60 images of transverse sections of each fibre type were quantitated. The data on mitochondria were expressed as percentages of the fibre volume.

RESULTS

Differentiation of fibre types

For the convenience of the present discussion, the fibre types described in the adult superior rectus muscle (Peachey et al. 1974) will be named here fibre type 'A' for small fibril granulated fibre, 'B' for large fibril granulated fibre, 'C' for lightly granulated fibre, 'D' for fibrillar white fibre, and 'E' for afibrillar fibre.

The distribution of the fibre types in the superior rectus muscle is neither random nor uniform. Certain fibre types have a tendency to be concentrated in particular regions of the muscle or to be absent from other regions of the muscle. Three regions of fibre type distribution can be distinguished in this muscle: orbital, global, and intermediate. The orbital region of the muscle is situated close to the orbital bone surface; the global region is close to the globe, or eyeball; and the intermediate region lies between these two regions. The orbital region contains only fibre types 'A' and 'B'. The intermediate region contains type 'C' in addition to the two orbital types. In the global region, there is a combination of the three fibre types mentioned above and the two additional types, 'D' and 'E'. The predominant fibre type in the global region is 'D'.

18-day embryos and newborn rats

The 18-day embryo muscle fibres looked alike with regard to their myofibrillar organization (Fig. 1). Differentiation and the assembly of the myofibrils were observed in all fibres, which did not exhibit the characteristic structural organization found in the adult muscle. The diameter of these muscle fibres was

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Fig. 4. Superior rectus of the 6-day-old rat, showing the differentiated 'A' and 'B' fibres (A, B). Note the abundant sarcoplasmic reticulum (Sr) around the myofibrils (Mb). M, mitochondria.

Fig. 5. A high-power electron micrograph of an 'A' fibre with well-delineated myofibrils (Mb) in I- and A-bands. Note that each myofibril is encircled by sarcoplasmic reticulum (Sr). Tt, transverse tubule; Z, Z-line.
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approximately 4.7 µm and did not change significantly until the first week of postnatal development. Free ribosomes and polyribosomes were abundant in these cells.

The superior rectus muscle of the newborn rat resembled the 18-day embryonic muscle. However, close examination revealed the onset of differentiation of large myofibrils characteristic of two fibre types ('B' and 'E') in the adult muscle. Scattered myotubes were present in the 18-day embryonic and newborn rat superior rectus muscle.

3- and 6-day rats

In the 3-day-old rats, a small population of differentiated 'A' fibres was observed in addition to 'B' fibres in the orbital region of the muscle. The latter with larger myofibrils and sparse sarcoplasmic reticulum (Fig. 2) were found to be more differentiated than the 'A' fibres. I-bands in the 'B' fibres were slightly delineated by well-differentiated sarcoplasmic reticulum. Scattered differentiating myotubes were observed at different stages of development during this period. Some contained abundant polysomes, scattered free myofilaments and, developing Z-lines along with the assemblage of myofilaments (Fig. 3). Rough endoplasmic reticulum with dense material was also observed in these myotubes. Desmosomes were present between young myotubes and differentiated muscle fibres.

The interesting feature of the 6-day muscle was the presence of well-developed 'A' and 'B' fibres throughout the body of the superior rectus. The myofibrils in A- and I-band regions of 'A' fibres were well delineated by sarcoplasmic reticulum unlike those of the 'B' fibres (Figs. 4, 5). Myotubes were rare beyond this developmental stage. Our quantitation indicated that 'A' fibres contained about 6% mitochondria by volume, whereas 'B' fibres contained about 4% by volume.

10- and 14-day rats

We observed an indication of differentiation that resulted in fibre type 'C', approximately in the intermediate region of the superior rectus muscle of the 10-day-old rats. The diameter of this fibre type measured approximately 8.6 µm. The myofibrils of the I-band region were provided with abundant sarcoplasmic

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Fig. 6. Fibre type 'C' in a 10-day-old rat superior rectus. The discrete myofibrils (Mb) in I-bands are surrounded by extensive sarcoplasmic reticulum (Sr). The myofibrils in A-bands are provided with less sarcoplasmic reticulum which are still in the process of differentiation. G, glycogen; L, lipid droplet; M, mitochondria; Tt, transverse tubule.

Figs. 7, 8. Longitudinal sections of fibre types 'A' and 'B'. The A- and I-bands are well delineated in fibre type 'A' by sarcoplasmic reticulum (Fig. 7), whereas in type 'B' (Fig. 8) the bands are not well delineated because of the paucity of the sarcoplasmic reticulum. Sr sarcoplasmic reticulum; Tt, transverse tubule.
Fig. 9. 'D' fibre in a 14-day-old superior rectus, showing layers of sarcoplasmic reticulum (Sr) around the myofibrils (Mb) and a regular occurrence of transverse tubules (Tt).
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reticulum, and the A-band region was differentiated into discrete units surrounded by differentiating sarcoplasmic reticulum (Fig. 6). Mitochondria were more than 11% by volume. During the second week of this developmental period, 'A' and 'B' fibres were fully developed in different regions of the muscle. Their diameters increased to approximately 7·6 \(\mu m\) and 6·6 \(\mu m\) respectively. The mitochondrial percent volume increased slightly in both fibre types. Figure 7 shows the 'A' fibres with A- and I-bands well delineated by sarcoplasmic reticulum. In contrast, the A-band region is almost a homogenous mass of actin and myosin filaments with sparse sarcoplasmic reticulum in 'B' fibres (Fig. 8). Between 10 and 14 days of development, fibre type 'D' differentiated in the global region of the muscle. The diameter of this fibre was similar to that of the 'C' fibre at this stage of development. The characteristic feature of this fibre was the highest content of sarcoplasmic reticulum, which was differentiated into more than one layer surrounding the myofibrils (Fig. 9). Both 'C' and 'D' fibres had undergone considerable further differentiation by the end of the second week. At this stage of development there was an indication of differentiation of fibre type 'E'.

3- and 6-week rats

During the third week of postnatal growth, fibre type 'E' differentiated in the global region of the muscle into fully formed fibres with characteristic features such as a homogenous mass of actin and myosin filaments in the A-band region, which did not contain discrete myofibrils because of the paucity of sarcoplasmic reticulum. The diameter of the fibre was approximately 6 \(\mu m\), which increased to 7 \(\mu m\) in the sixth and final week of the study. The sparse mitochondria were smaller than in other fibres (Figs. 10, 11) and constituted 4·5% of the fibre volume. The I-band region also contained very little sarcoplasmic reticulum. During the third to sixth week of development, the other fibre types described earlier exhibited extensive differentiation of sarcoplasmic reticulum, transverse tubules and mitochondria (Fig. 12). The superior rectus muscle of the 4-week-old rat differentiated into all five fibre types, which were in turn undergoing further elaboration of their organelles during subsequent developmental periods. Our quantitations indicated that these fibres justified their identity as distinct fibre types with the characteristic features. The diameters of the fibre types 'A' and 'B' were approximately more than 8 \(\mu m\) and 6·6 \(\mu m\) respectively during the sixth week of their development. Fibre types 'C' and 'D' were approximately more than 10 \(\mu m\) and 12 \(\mu m\) in diameter during this terminal part of our study. The mitochondria content of the fibre types 'A', 'B', 'C', and 'D' during this terminal period was approximately 16, 12, 10, 7% by volume. Fibre type 'E' did not show any significant increase in mitochondrial content other than its initial period of development.
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Differentiation of neuromuscular junctions

The differentiation of neuromuscular junctions was observed in the 3-day postnatal muscle. The junctions at this stage of development were small and did not exhibit postsynaptic folds (Fig. 13) as observed in the adult physiological slow fibres (Hess, 1967; Harker, 1972). Also, the presynaptic nerve ending did not contain abundant synaptic vesicles and mitochondria. The muscle fibres involved in the establishment of neuromuscular junctions showed the differentiating characteristic features of 'B' fibres, which contained large myofibrils with scanty sarcoplasmic reticulum. In the first week after birth, a second type of neuromuscular junction with postsynaptic folds was observed among those fibres which exhibited characteristic features of 'A' fibres (Fig. 14). In addition, the simple type of junction mentioned above was also present at this developmental stage. It is interesting to note that as the development of the rat proceeded, both types of junctions became larger and contained more synaptic vesicles, mitochondria and microtubules than those of the earlier stages (Fig. 15). During the second week of development, neuromuscular junctions with extensive postsynaptic folds were found to be differentiated in association with fibres which exhibited structural features of fibre types 'C' and 'D'. The postsynaptic folds in these fibres were deeper (Fig. 16) than those of the type 'A' fibres. In the third week of development, a very simple type of neuromuscular junction with few or no postsynaptic folds was observed on the 'E' fibre. With the exception of those associated with type 'B' fibres, the presynaptic nerve endings on type 'E' fibres differentiated with fewer synaptic vesicles and mitochondria than those of the other fibre types.

DISCUSSION

It is now well known that mammalian extraocular muscles contain several structural fibre types, which differ from one another with respect to diameter, myofibrillar size, sarcoplasmic reticulum, transverse tubule and mitochondrial content. In addition, the neuromuscular junctions of these fibres differ from one another in size and extent of elaboration of postsynaptic folds. The present study on one of the developing extraocular muscles, the superior rectus of rats, revealed that the five fibre types differentiated gradually as the development of the animal proceeded. The orbital fibres 'A' and 'B' differentiated with their

Fig. 10. Transverse section of a differentiated 'E' fibre in a 3-week-old superior rectus of a rat. The large continuous myofibril is evident in this micrograph. Note the paucity of sarcoplasmic reticulum in A- and I-bands. M, mitochondria.

Fig. 11. Longitudinal section of an 'E' fibre in the 3-week-old superior rectus of the rat. Note the paucity of the sarcoplasmic reticulum (Sr) and broad, poorly delineated A- and I-bands.
Fig. 12. Longitudinal section of a fibre type 'C' in a 4-week-old superior rectus of the rat, showing well-differentiated sarcoplasmic reticulum (Sr) and transverse tubules (Tt).

Fig. 13. Neuromuscular junction of a differentiated 'B' fibre in a 3-day-old superior rectus of the rat is shown (arrows). Note that the presynaptic nerve ending does not contain many synaptic vesicles and mitochondria. Sv, synaptic vesicles.
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Fig. 14. The neuromuscular junction of the fibre type 'A' in a week-old superior rectus of the rat. Note the differentiated postsynaptic folds (arrows). M, mitochondria; Sc N, Schwann cell nucleus; Sr, sarcoplasmic reticulum; Sv, synaptic vesicles.
Fig. 15. The neuromuscular junction of the fibre type ‘B’ in a 10-day-old superior rectus muscle of the rat. The junction became larger than the 3-day-old rat (Fig. 13). Note the absence of postsynaptic folds. M, mitochondria; Rer, rough endoplasmic reticulum; Sc N, Schwann cell nucleus; Sv, synaptic vesicles.
conspicuous structural features ahead of other fibres in the muscle. In embryonic and newborn rats, all fibres looked alike because of their undifferentiated state. The differentiation of myofibrils and sarcoplasmic reticulum enabled us to identify these fibres initially. Later on, the differentiation of neuromuscular junctions and other structural features reinforced our identification.

The interaction of nerve and muscle in the formation of synapses has been studied recently (Fischbach & Cohen, 1973; Famborough, 1974; Frank & Fischbach, 1979; Harris, 1981a, b, c). Acetylcholine receptors were found on many uninnervated myotubes. However, further growth of these receptors and the maintenance of their position in a narrow band across the midline of the
muscles depend on neural regulation (Harris, 1981). In all cases where individual myotubes were examined before and after synapse formation, ingrowing axons induced new clusters of receptors rather than seeking out pre-existing clusters. Synapses can form at active growth cones within 3 h of nerve–muscle contact, and new receptor clusters can appear beneath neurites within a few hours (Frank & Fischbach, 1979). Harris's work (1981c) supports the view that the pattern of innervation is determined by the developing muscle, which directs the placement of the junctions.

There are two views concerning the role of innervation in the growth and differentiation of muscle fibres. One view suggests that muscle growth and differentiation are not dependent on innervation (Zelená, 1962; Stewart, 1968, 1972; Gutmann, Schiaffino & Hazlikova, 1971). This apparently reflects an intrinsic capacity of muscle tissue to differentiate in the total absence of nerve. It has also been suggested that extrinsic non-neural factors, such as the stretch caused by skeletal elongation, may be responsible for muscle growth in the denervated limb (Stewart, 1968). The role of tension in muscle development is known (Stewart, 1972), and it has been shown that passive tension, in the absence of neural mediation, may also have a part in compensatory muscle hypertrophy (Gutmann et al. 1971). However, recent studies of Harris (1981a) indicate that the myogenic activity in aneural muscles is at least as strong as in muscles with a tetrodotoxin-induced nerve block and yet does not maintain normal development.

The other view suggests that the differentiation of muscle fibres is dependent upon innervation (Guth, 1968; Hanzlikova & Schiaffino, 1973; Schiaffino, Pierobon-Bormioli & Aloisi, 1974; Harris, 1981a, b, c). Schiaffino et al. (1974) studied the effect of foetal denervation on the differentiation of rat skeletal muscle fibres and found that myofibrillogenesis and sarcotubular differentiation were at first basically unchanged in denervated muscle fibres. However, the differential changes in the fine structure of the various fibre types, which in the normally innervated muscles took place soon after birth, were completely prevented by foetal denervation. This indicates that neuromuscular interactions are apparently required for the differentiation of muscle fibre types. Harris (1981a) showed that both innervation and muscle electrical or contractile activity were essential for the normal development of new muscle fibres. Without innervation, only primary myotubes were formed, and secondary myotubes, which gave rise to 80% of the fibres in an adult muscle, failed to appear. The contraction of muscle was also required for generation of new fibres. If the muscles were paralysed, and even if they were innervated, development of secondary myotubes rapidly ceased. These findings suggest that innervation may have a trophic action as well as a part in activating muscle contraction.

Although our studies indicated that the initial phenotypic features, such as organization of myofibrils, sarcoplasmic reticulum and mitochondrial content of the fibre types, differentiated before the establishment of the neuromuscular
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junctions, we cannot rule out the possibility of the presence of neuromuscular interactions at earlier stages of development of the muscle. However, it is quite possible that the sequential differentiation of muscle fibre characteristics was genetically programmed, and the innervation further elaborated the distinguishing features of these fibre types.

During the development of the superior rectus muscle from 18 days onward, we did not observe any pattern of myotube assembly except for some scattered myotubes among the differentiated fibres which were alike at the initial stages of development. Myotubes were rare in the muscle after the first week of postnatal development.

Although all muscle cells looked alike at the initial stages of development, it cannot be said that they originated from a single population of myotubes. Since the cells at the embryonic and early postnatal stages of development were involved in the synthesis of contractile proteins and other components of the cells, they did not exhibit particular features of a specific fibre type. This does not rule out the possibility for the presence of more than one population of myotubes which would give rise to several different fibre types. It is also possible that a single population of myotubes differentiated into muscle fibres which were all alike and that later they differentiated into fibre types with the establishment of the neuromuscular contacts, as the animal matured.

The postnatal rats usually opened their eyelids between the tenth and fourteenth days of their development. At least two fibre types ('A' and 'B') differentiated before the tenth day. The indications for the differentiation of the rest of the fibre types were apparent between the tenth and fourteenth days. The programmed differentiation of the fibre types with their neuromuscular junctions probably reflects the different metabolic and electrical needs of this muscle to carry out diverse functions, such as critical focusing, accommodation, movements, etc. required for the daily life activities of rats, especially after the opening of their eyelids.

The present study has demonstrated the sequential differentiation of fibre types in the superior rectus muscle. It is interesting to note that although the fibre types exhibited all of the characteristic structural features by the sixth postnatal week, they did not yet attain the maximum fibre diameter which we observed in the adult rat. However, it is surprising how little is known about the factors regulating the differentiation and adaptive responses of the muscle fibre types. The present state of our knowledge of this aspect appears to be primitive. We believe that, unless the differentiation of fibre types at the molecular level is studied to trace in detail the pathway from the action of the gene to the final expression of its phenotype, the integrative factors, such as neural trophic influences controlling fibre differentiation, will not be understood.

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