Prenatal Morphogenesis of Primate Extraocular Muscle: Neuromuscular Junction Formation and Fiber Type Differentiation

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Extraocular muscle represents a distinctive class among mammalian skeletal musculature in exhibiting the full range of muscle fiber type variability found in vertebrate species. To better understand the basis for the unique structural/functional diversity of extraocular muscle, the ontogeny of lateral rectus muscles was studied in Macaca nemestrina fetuses of 62–156 days gestation using light and electron microscopy. At E62, myotubes and myofibers are evident, but fiber-type differentiation has not yet occurred and neuromuscular junctions are primitive. By E92, presumptive singly and multiply innervated fiber types could be distinguished on the basis of myofibril delineation. Like other skeletal muscles, extraocular myogenesis proceeds through at least two generations of myofibers. All primary and secondary myofibers were generated and were maturing by E121. The phylogenetically “old” global multiply innervated fiber type was the first to attain adult form. This was followed by maturation of global layer singly innervated fiber types, which are developed by E156, except for attainment of definitive size and mitochondrial content. Orbital layer fiber types, particularly the orbital singly innervated fiber, are the last to mature. Neuromuscular junction maturation paralleled the changes observed during fiber-type differentiation. In summary, the sequential development of their constituent muscle fiber types may reflect the functional pressures the extraocular muscles are exposed to by maturing visual and visuomotor systems. In particular, ontogenic and phylogenic changes observed in the orbital singly innervated fiber type may have direct implications for the types, range, and precision of eye movements used by different species and at different gestational ages. Invest Ophthalmol Vis Sci 33:657–670, 1992

The present study examined the prenatal development of fiber types in the extraocular muscles of a primate. These muscles represent a distinctive class among mammalian skeletal muscles. Extraocular muscle fiber types do not easily fit the traditional fiber classification schemes. In addition, these fiber types include multiply innervated fiber types rare in mammalian skeletal muscle but common in birds and amphibians. The differences between these muscles and other skeletal muscles may be related to their embryological origin from isolated mesenchymal condensations of neither somite nor branchial arch origin. However, it is generally believed that the plasticity of a muscle is dictated by the plasticity of its motoneuron pool. Thus, the unique fiber type composition of these muscles (for review, see Spencer and Porter1) may be, in part, related to the diversity of eye movement systems and the high discharge rates of oculomotor motoneurons.2

Extraocular muscles are responsible for reflexive eye movements with velocities as low as <1°/sec to saccadic movements of velocities up to 600°/sec. At the same time, they maintain precise interocular alignment to prevent diplopia. Also, oculomotor motoneurons exhibit sustained firing rates of up to 300/sec and phasic discharges of up to 400/sec. These values are an order of magnitude higher than that seen for other somatic motoneurons. Given that muscle metabolic and contractile characteristics are highly correlated with motoneuron physiological characteristics, the unusual properties of oculomotor motoneurons probably play a role in shaping the definitive muscle fiber types. The relative contribution of myogenic factors and neurotropic regulation of gene expression in fiber type differentiation, however, has yet to be clearly established for any skeletal muscle.

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Supported by USPHS grant R01 EY05464 from the National Eye Institute, Bethesda, Maryland, and a grant from the University of Kentucky Medical Center Research Fund, Lexington, Kentucky.

Submitted for publication: August 9, 1991; accepted October 22, 1991.

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To better understand the unique features of the extraocular muscles, this study examined their prenatal development in a primate model to establish the temporal relationship between axon and axon terminal maturation and fiber type differentiation. Present findings indicate that the phylogenetically "old" global multiply innervated fiber type is the first to develop. This is followed by maturation of global layer singly innervated fiber types, which are developed by birth except for attainment of definitive size and mitochondrial content. Orbital layer fiber types, particularly the orbital singly innervated fiber, are the last to mature. Ontogenic and phylogenetic changes in the orbital singly innervated fiber type may directly impact the range and precision of eye movements. Preliminary results have been presented in abstract form elsewhere.3

Materials and Methods

Tissue was obtained from timed pregnant monkeys (Macaca nemestrina) at the breeding colony at the Regional Primate Research Center, University of Washington, Seattle, WA. All procedures involving animals adhered to the ARVO Resolution on the Use of Animals in Research. Monkey gestational ages were accurate to within a maximum of ±5 d. Fetuses were delivered by Caesarean section at 62, 92, 121, 135, and 156 days of gestation (embryonic days are designated as E, postnatal days as P; term in this species is approximately El66) and were anesthetized with Nembutal intraperitoneally. Two fetuses were male, two were female, and one was undetermined. Most animals were perfused with Ringer's/dextrose solution containing 2% heparin, followed by fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer. Muscles from the E62-day case were fixed by immersion. Following fixation, muscles were shipped to Kentucky for further processing. Lateral rectus muscles were washed in cold phosphate buffer and cut transversely at 50 μm using a Smith-McIlwain tissue sectioner (Brinkman, Westbury, NY). Muscle sections then were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, stained en bloc with 0.05% uranyl acetate in maleate buffer, dehydrated in a graded series of methanols, followed by propylene oxide, and embedded in epoxy resin. Semithin sections were cut with an ultramicrotome, stained with 0.1% paraphenylenediamine, and examined with phase contrast optics. Ultrathin sections containing representative regions of each muscle were cut, picked up on 135 mesh honeycomb grids, and stained with uranyl acetate and lead citrate. Sections were examined and photographed by using a Hitachi H-7000 electron microscope. For comparison, adult extraocular muscles were available from other studies in this laboratory.5

Results

Muscle Fiber Morphology in Adult Extraocular Muscle

Adult monkey lateral rectus muscles exhibit six distinct fiber types that do not conform to any of the routine muscle classification schemes.1,4 Muscles contain a c-shaped orbital layer that lies away from the eye and a global layer found in relation to the eye and optic nerve. The extraocular muscle fiber-type classification of Spencer and Porter1 is used in the present report. The distinction between singly and multiply innervated fiber types was based upon the longitudinal separation of neuromuscular junctions on individual fibers. The orbital layer contains one singly (SIF) and one multiply (MIF) innervated fiber type. The orbital SIF (Fig. 1D) exhibits properties typical of fast twitch, fatigue-resistant fibers and is characterized by extensive mitochondrial aggregates and a surrounding capillary bed. Orbital MIFs (Fig. 1D) are smaller in diameter, present fewer mitochondria and distributed neuromuscular junctions, and exhibit myofibril characteristics that vary along the length of individual fibers.6 The orbital MIF resembles the avian (twitch) type with distributed neuromuscular junctions. The global layer contains three SIF types and an additional MIF type distinct from that of the orbital layer (Fig. 2D). Global SIFs are distinguished largely according to number, size, and distribution of mitochondria. All global SIFs are fast twitch (among the extraocular muscles, slow twitch fibers are found only in the levator palpebrae superiors), and include global red, global intermediate, and global white SIFs. Because of the presence of the extensive mitochondrial aggregates, the global red SIF closely resembles the orbital SIF. Global MIFs resemble amphibian tonic (nontwitch) fibers with their distributed neuromuscular junctions and poorly delineated myofibrils. The specific details of adult neuromuscular junction morphology are described in detail elsewhere.1

Early Developmental Events

At the earliest time studied (E62), monkey lateral rectus muscles did not yet exhibit the layered organization that characterizes the adult but instead contained primitive myofibers and myotubes scattered among a loosely organized connective tissue matrix and sparsely distributed, thin-walled vascular elements (Fig. 3A). Activate fibroblasts were abundant. None
Fig. 1. Electron photomicrographs of orbital layer of monkey lateral rectus muscles at E92 (A), E121 (B), E156 (C), and adult (D). At E92 (A), fibers are small in diameter, show little evidence of subtype formation, and the layer contains only scattered vascular elements (v). Secondary generation myofibers are seen in association with the larger primary ones. With the exception of increased vascularization, little change is evident in this layer by E121 (B). No associations of primary and secondary myofibers are evident at this stage. While orbital layer fiber types continue to be underdeveloped just before birth (E156, C), this layer is recognizable by means of the higher mitochondrial content of orbital singly innervated fibers and the increased microvascular network (c). Orbital multiply innervated fibers (M) are small and poorly developed. In the adult (D), the orbital singly innervated fiber type is characterized by extensive mitochondrial aggregates and dense capillary bed (c). At neuromuscular junctions, axons (a) encircle individual fibers. Multiply innervated fibers (M) are small and contain fewer, small mitochondria and poorly delineated myofibrils. Original magnifications × 2250 (A, B, C), × 1300 (D).
Fig. 2. Electron photomicrographs of global layer of monkey lateral rectus muscles at E92 (A), E121 (B), E156 (C), and adult (D). E92 monkey (A) exhibits early appearance of global multiply innervated fiber type (M), distinguished on the basis of myofibril size. Vasculature is sparse, and scattered, small-diameter unmyelinated axons (a) are present. By E121 (B), the darker matrix of global multiply innervated fibers (M) easily distinguishes them from singly innervated types. Fibers are larger in diameter and vascular bed development is higher than in the orbital layer at this same stage. Axons are largely unmyelinated, although some scattered myelinated axons are present (a). Resolution of global singly innervated fiber subtypes is possible by E156 (C), although their mitochondrial content is not yet that of the adult. Myelinated axons (a) are distributed throughout the muscle. In the adult (D), singly innervated fiber types may be distinguished on the basis of the number, size, and distribution of mitochondria. Capillaries (c) ring the global red singly innervated fiber type. Original magnifications ×2250 (A, B, C), ×1500 (D).
Fig. 3. Electron photomicrographs of early developmental events in monkey lateral rectus muscles. Two putative primary generation myotubes are coupled (between arrows) in E62 monkey (A). Similar coupling is apparent (B) between putative primary (at right) and secondary (at left) generation myofibers. Well developed A and I bands are evident in the primary generation cell. Note that the same basal lamina surrounds both cells. High-magnification micrograph (C) of E62 myofiber indicates both the early sarcomeric organization of myofilaments and triadic contacts between I-tubules (arrow) and sarcoplasmic reticulum. Myofibrils are poorly delineated at this stage. Comparison with myofibrillar organization in an adult global singly innervated fiber (D) is illustrated (arrow indicates a triad). Original magnifications ×9000 (A), ×22,500 (B), ×31,500 (C), ×31,000 (D).
of the six extraocular myofiber types could be resolved at this gestational stage. Individual myofibers or myofiber clusters were surrounded by a wispy basal amina and few collagen fibrils were present in the interstitial space. Tight clusters of myofibers, with individual fibers often linked by gap junctions, were a prominent feature at the earliest gestational ages studied (Fig. 3A). The majority of muscle cells were multinucleate myotubes or myofibers, containing slightly infolded euchromatic nuclei frequently central in location.

In the E92 monkey, the tight cell clusters were a less obvious feature, but, when present, they typically contained early and late differentiating myofibers, with the smaller and more primitive secondary myofibers often sending cytoplasmic projections into presumptive primary generation myofibers (Fig. 3B). This contrasts with the E62 case, at which time the myofibers that made up individual clusters were at similar developmental stages, and all were likely primary generation cells. Myonuclei contained more heterochromatin and prominent nucleoli. At this stage, it was not yet possible to clearly distinguish the characteristic muscle layer organization. It was possible only to identify those general regions of the muscle primordium that ultimately would give rise to orbital and global layers. Connective tissue elements remained limited, as much of the intercellular space was filled with a loosely organized matrix, with collagen bundles particularly evident in relation to neurovascular elements. Only by E135 to E156 was the interstitial connective tissue elaborated to the point that it resembled adult form. At E92, the microvascular network remained relatively sparse, with even the largest vessels consisting of an endothelium and a maximum of 1-2 layers of smooth muscle.

At E62, the basic sarcomeric organization of actin and myosin filaments, consisting of hexagonal arrangement of actin around each myosin filament, was already in place (Fig. 3C). The interlocking of actin filaments at the Z-line did not yet appear to be complete. The early myofibrils were loosely defined, often surrounded by clear cytoplasmic areas that presumably allowed for their further expansion in size. Likewise, while the extent of internal membrane system development was slight, clear triadic contacts between elements of the t-tubule system and sarcoplasmic reticulum were evident in some fibers (compare Fig. 3C with the adult morphology shown in Fig. 3D).

An active Golgi apparatus was present in many fibers. By E92, myofibrils were conspicuously larger, with little or no surrounding areas of vacant cytoplasm, and were better delineated by elements of the internal membrane system. Mitochondria, with well developed cristae, were evident as early as E62.

**Fiber Type Differentiation**

Myofibers in 62-day fetal extraocular muscles were homogeneous in appearance, with individual fibers exhibiting similar mitochondrial content and degree of myofibril delineation. Initial expression of any of the distinctive characteristics of extraocular muscle fiber types was first apparent at E92 (Figs. 1A and 2A). Singly and multiply innervated types then could be distinguished as myofibrils were well delineated by internal membrane systems in the former and remained large and poorly delineated in the later. The lack of divergence in mitochondrial content at this time precluded identification of the distinctive SIF subtypes.

By E121, primary and secondary generation myofibers could no longer be distinguished and the resolution of SIFs from MIFs was clear for both extraocular muscle layers. In particular, global MIF morphology began to approach the degree of maturation of myofibrils and internal membrane systems evident in the adult (Fig. 2B). At this time, this fiber type also exhibited the higher electron density of myofibrils that characterizes the adult. The global MIF then was the first type to attain definitive form and, at this stage, was disproportionately large compared to the singly innervated types. The occurrence of significant elaboration in internal membrane systems and in the number, size, and distribution of mitochondria marked the early stages of appearance of SIF subtypes. Throughout the time frame of study, however, orbital fiber types lagged behind those of the global layer in obtaining adult characteristics. Concurrent with fiber type specialization, and likely related to increased metabolic demands, the microvascular network matured to a level that was considerably more extensive than at earlier stages. With increased fiber size, intercellular space was markedly reduced.

Changes in oxidative capacity were observed in SIFs by E135. In particular, the increased mitochondrial content of orbital SIFs allowed better differentiation of extraocular muscle layers at this stage. Mitochondria were largely distributed singly. Only occasionally were they seen to demonstrate the clustering that characterizes this fiber type in the adult. Taken together, observations at E135 suggested that the orbital layer continued to lag behind global layer fiber types in size and elaboration of internal ultrastructure.

The basis for functional and metabolic differences between the orbital and global layers were clearly evident by E156 (Figs. 1C and 2C). While all fiber types were qualitatively smaller than in the adult, global layer intermediate and pale SIFs now were easily distinguished as a result of variations in mitochondrial
content that presumably translate into fatigability differences. Orbital MIFs remained small and poorly developed at this stage, only to mature rapidly in the early postnatal period. At E156, the global red and its counterpart orbital SIF types remained immature in terms of obtaining definitive mitochondrial content. Continued elaboration of vascular elements resulted in an extensive capillary network in association with orbital SIFs. Commensurate increases in the mitochondrial content of this fiber type led to the initiation of formation of the central and subsarcolemmal clusters that characterize these fiber types. These two highly fatigue-resistant fiber types were, however, the last to attain adult form, reaching their definitive anatomical profile in the first few postnatal months.

Neuromuscular Junction Maturation

The early nerve bundles seen in the E62 animal contained small (approximately 1–3 μm diameter) axons, but, surprisingly, there were few growth cones observed at this or any later stage (Fig. 4B). The majority of axons were clustered into discrete bundles surrounded by the processes of presumptive Schwann cells. (High polyribosome and modest rough endoplasmic reticulum content and the presence of a basal lamina distinguished Schwann cells.) Axons contain substantial membranous material, presumably smooth endoplasmic reticulum, and relatively few neurotubules and neurofilaments. The individual axons were not discretely isolated, but typically were in direct contact with one another. Very few presumptive terminals were observed in direct anatomical contact with myofibers. Rather, most "terminals" were at distances >100 nm from the sarcolemma with synaptic clefts of irregular width (Fig. 4A). Occasionally, Schwann cells and myofibers were attached by junctional complexes (Fig. 4A). Synaptic vesicles were clear and spherical, with a few dense core vesicles, and were sparsely distributed in preterminal and terminal axons. Thickening of the sarcolemma immediately adjacent to nerve terminals represented the only postjunctional change observed at this time.

By E92, most axons were isolated from one another by Schwann cell processes, and nerve bundles were well defined by connective tissue elements (Fig. 4D). Occasional bundles of small diameter, presumably preterminal axons still lacked the separation from each other. Axons exhibited the adult complement of organelles, including organized bundles of neurofilaments with interspersed neurotubules. Myelination of some axons was encountered only near point of entry of nerve into the muscle. Even then, only a very thin layer of myelin was present. Elongated neuromuscular contact zones, usually with overlying Schwann cells, were characteristic of junctions associated with presumptive SIFs at this developmental stage (Fig. 4C). Synaptic contacts with putative MIFs were smaller and did not occupy such an extensive portion of the fiber surface. Neuromuscular junctions were seen in association with presumptive primary generation myofibers only, as terminals were not observed on the small, primitive cells that were tightly associated with the primary myofibers. Terminals were filled with spherical, clear synaptic vesicles and small mitochondria. The beginnings of postjunctional specializations, including aggregations of mitochondria and highly infolded myonuclei, were observed occasionally at junctions associated with SIFs. Postjunctional folding of the sarcolemma was not noted at this or any other prenatal stage.

At E121, the terminals on global SIFs had become more discrete, as the elongated nerve/muscle appositions seen at E92 were reduced to focal contacts. At this point, however, presynaptic elements associated with singly innervated fibers still lay on the fiber surface and were not yet embedded in sarcolemmal depressions. In keeping with the degree of maturation of the global MIF structural characteristics, the neuromuscular junctions associated with this fiber closely resembled those of the adult (Fig. 5). Compared to the E92 case, myelin sheaths of intramuscular axons were thicker and were no longer restricted to the nerve entry zone, but extended farther into the substance of the muscle.

Further changes in neuromuscular contacts and, in particular, postsynaptic elements, characterized E135 monkey extraocular muscles (Figs. 6A and 6B). While neuromuscular contacts continued to become more focal in appearance (opposed to the elongated contacts of E92), stacking of preterminal synaptic elements upon one another was first evident in some junctions at this stage. Some of the neuromuscular junctions associated with global SIF types showed embedding of the presynaptic element in deep depressions of the sarcolemma. The distinctive spiraling of nerve terminals around orbital SIFs seen in the adult also became apparent by this stage. Massive aggregations of highly infolded myonuclei were noted at the junctional region of singly innervated fiber types (Fig. 6A). Postjunctional accumulations of mitochondria also became evident in some SIFs at this stage.

E156 day neuromuscular junctions were structurally mature in virtually all respects, except for elaboration of postjunctional folds in global SIF types (Fig. 6C). Such folding typifies the neuromuscular junctions of adult global intermediate and pale SIF types (Fig. 6D). This feature develops within the first three postnatal months.
Fig. 4. Electron photomicrographs of early neuromuscular junctions (A, C) and intramuscular nerves (B, D). At E62 (A), developing synaptic terminals (s) closely approach developing primary myotubes. Scattered synaptic vesicles are evident in putative terminals. Schwann cells (Sch) are associated with developing neural elements, and, occasionally, form junctional complexes with muscle (arrow). At this stage, intramuscular axons are small and unmyelinated (B). Schwann cell processes (arrows) surround bundles of axons, but do not enclose individual axons. An early myotube, characterized by central nuclei (n), exhibits a closer synaptic contact (s) and postsynaptic sarcolemmal thickening at E92 (C). By this stage, individual axons (a) are surrounded by Schwann cell processes, and nerve bundles are enclosed by collagen and fibroblast processes (D). ×20,700 (A), ×16,500 (B), ×16,000 (C), ×4600 (D).
Discussion

The extraocular muscles arise from maxillomandibular and premandibular condensations of mesoderm that appear within the orbit. The maxillomandibular condensation gives rise to muscles innervated by the abducens and trochlear nerves, while the premandibular condensation forms those muscles innervated by the oculomotor nerve. At the cellular level, the generation of the extraocular muscles follows the same general stages described for other skeletal muscles: mesenchymal cell, early myoblast, myoblast, fusion of myoblasts, myotube, and mature myofiber. Whether germinative cells are pluripotential, subject to neural or environmental influence, or exist as distinct myoblast lines already destined to form singly or multiply innervated fiber types has not yet been determined for extraocular muscle. Distinct myoblast lines have been described in skeletal muscles of birds. The present study has established the sequential maturation of fiber types in lateral rectus muscles of the monkey from the myotube/early myofiber stage to birth. Like other skeletal muscles, the extraocular muscles are generated asynchronously from at least two waves of myogenesis responsible for formation of primary and secondary fibers. This report is concerned with the maturation of adult eye muscle fiber types from the ultrastructurally homogeneous population of cells present at E62. Global layer fiber types, beginning with the global MIF, matured earlier than fiber types in the orbital layer. The potential correlations between the sequence of events in morphogenesis of the extraocular muscles and maturation of visuomotor systems are discussed below.

At the earliest stage examined (E62), the extraocular muscles consisted of a homogeneous population of myotubes and myofibers. The lack of resolution of ultrastructurally defined fiber types at this stage does not, however, exclude the possibility of early divergence in cell lineage, as individual cells already may be committed at the level of gene expression. Tight coupling between primary myotubes was characteristic of E62 monkey extraocular muscles. The presence of myofiber linkage by way of gap junctions is typical of developing skeletal muscle and is hypothesized to provide for spread of activation between innervated and noninnervated myotubes. Like other skeletal muscles, second generation myofibers in extraocular muscle appear to form only through associations with primary generation fibers (secondary fibers are generated in monkey lateral rectus muscles between E62 and E92). At E92, multiple secondary myotubes are coupled to individual primary myotubes, all contained within the same basal lamina. Given the ab-
Fig. 6. Electron photomicrographs of neuromuscular junctions associated with singly innervated fiber types at E135 (A, B), E156 (C), and adult (D) stages of development. E135 terminals (A, B) are characterized by dense aggregations of terminal boutons (s) and myonuclei (n). Many nerve terminals are well filled with synaptic vesicles, and postsynaptic accumulations of mitochondria have become evident (B). A nerve terminal associated with a global white singly innervated fiber is mature at E156, except for the formation of subjunctional folding in the sarcolemma (C). Subjunctional folds are clearly evident (arrows) in this same fiber type in the adult (D). ×6900 (A), ×11,500 (B), ×16,000 (C), ×11,500 (D).
sence of neuromuscular contacts on secondary generation myotubes, the presence of such coupling suggests that neural activation, via gap junction contacts with the primary myotube, may be important to subsequent maturation of the cell. Such secondary generation fibers depend greatly upon innervation and muscle contraction, as loss of either halts their formation. The present report's findings in the monkey suggest that extensive formation of new myofibers continues until between E92 and E121. Thus, the loss of the fiber-fiber coupling by E121 may reflect the status of maturation of motor innervation and onset of independent function in all developing myofibers.

For most skeletal muscles, the anatomical and contractile properties of slow twitch (type I) fibers develop first. Relating extraocular muscles to other skeletal muscles, Lannenstrands and coworkers indicate that, structurally and functionally, cat extraocular muscle slow-fiber systems develop earlier than fast systems. These data are consistent with present observations in that the global MIF precedes all other types in attainment of adult characteristics. However, the slow fiber systems in extraocular muscle, which are unusual in presentation of multiple innervation, are neither the structural nor functional equivalent of typical skeletal slow twitch fibers. The early development of a slow, tonically contracting fiber is difficult to interpret, particularly because the functional role of this fiber type in the adult is not well understood. However, because this fiber type is a component of the principal sensory receptor found in extraocular muscle, it is plausible that early maturation of the global MIF allows proprioceptive information to be used in visual system development.

Global SIFs mature rapidly in the late prenatal period. Although their mitochondrial content does not attain adult levels until after birth, ultrastructural characteristics were such that the three global SIF types could be discerned by E156. The observation that orbital layer fiber types mature last is consistent with the hypothesized role of orbital SIF in oculomotor control. The exceptionally high mitochondrial content of orbital SIFs is consistent with elevated fatigue resistance, and thus is consistent with a sustained level of activity in eye position maintenance. Indeed, motor units in the orbital layer are recruited early during initiation of an eye movement. As oculomotor systems mature, the increased fatigue resistance of this fiber type presumably is an adaptive response to the development of disjunctive eye movements and fusion, which are particularly important to primates. Consistent with this view, across mammalian species, the orbital SIF exhibits its highest mitochondrial content in the monkey and man.

Muscle fiber differentiation and motor unit development are known to parallel one another. Indeed, neural influence upon muscle properties apparently can occur at any of several stages. Given the primitive state of neuromuscular junctions at E62, it is likely that the early differentiation of basic muscle structure can occur in the absence of innervation. In other muscles, the lack of this early activity leads to an arrest in formation of secondary fibers. Likewise, denervation at birth interferes with the transition from "slow" to "fast" characteristics that typifies the development of most skeletal musculature. However, later differentiation of the definitive fiber type composition of these muscles most likely requires not only innervation, but probably the particular patterned activity imposed by the motor control system (see below). Based on the complex nature of extraocular muscle myosin expression, which includes the presence of embryonic and neonatal isoforms as well as cardiac and extraocular muscle specific types, it is likely that the role of neurogenic factors in the regulation of gene expression in these muscles is significant. Extraocular muscles, however, show evidence of possessing a high degree of intrinsic plasticity, including the expression of multiple developmental myosin isoforms in the adult, extraordinarily high resistance to denervation atrophy and development despite the absence of the globe in an anophthalmic mouse model. Thus, the potential myogenic origin of some of their unique features cannot be ignored.

Species Comparisons

The early events observed in developing monkey extraocular muscles closely paralleled those seen in limited studies of human fetuses. Muscles of the E62 monkey closely resembled those of an approximately E70 human fetus in presenting tight clusters of myotubes/myofibers linked by gap junctions and axon bundles that were wrapped simply by Schwann processes. Likewise, the tight associations of primary and secondary myofibers apparent in the E92 monkey also were seen in E84 and E105 human fetuses. An E161 human fetus differed from the later stages of monkey extraocular muscle development. The primary-secondary myofiber associations were present over a longer time than was observed for the monkey. Together, these data suggest a longer proliferative phase in man. Near-term human fetal extraocular muscles, comparable to the later stages examined in the present study, have not been previously studied.

The monkey exhibits a greater degree of fiber-type differentiation during the prenatal period than either of the experimental animals (cat and rat) in which extraocular muscle development has been stud-
Moreover, muscle morphogenesis is much more compressed to within the perinatal and early postnatal periods in the cat and rat. Later maturation of extraocular muscle in these species may be related to timing of eyelid opening (open at birth in monkey, while open about P14 in cat and rat). Extraocular muscles of an E62 monkey look much like those of E18 rat in that myofibers are homogeneous, lacking differentiation of any of the definitive adult fiber types. The similarities appear to end there. In contrast to the monkey, development of the orbital layer precedes that of the global layer in the rat. Moreover, these authors indicate that the global MIF, the first type to be defined in the monkey, is the last to mature in the rat.

In contrast, development of the layered organization of cat extraocular muscles closely resembles that of the monkey, with global preceding orbital. The two species differ in the overall time course of myogenesis (many events that are prenatal in the monkey occur in the early postnatal period in the cat) and in the ultimate degree of mitochondrial development in the orbital SIF (with higher mitochondrial content in monkey). In summary, species comparisons of extraocular muscle ontogeny suggest that the sequential development of fiber types is conserved in lateral-eyed organisms but may follow a different sequence in lateral-eyed species. If such developmental differences are consistently observed in comparisons of animals with frontally and laterally placed eyes, a strong tie can be made between muscle morphogenesis and requirements for interocular alignment, fusion, and vergence eye movements.

In general, neuromuscular junction formation appears to follow similar paths in rat, monkey (present findings), and man. Events occurring during synaptogenesis did not differ markedly from those previously described in other skeletal musculature. Initial contacts are small and evolve into much elongated contacts that ultimately are reduced to the form seen in the adult (eg, P7 rat exhibits the same elongated contacts seen in E92 monkey, although these later become focal in both species). Similar to the situation in the monkey, postjunctional folds form in the postnatal period, but only in association with particular fiber types. The restriction of postjunctional folds to particular fiber types suggests that their formation is regulated by activity levels. Using an extraocular muscle model, Sohal argues that postjunctional specializations may develop in the absence of innervation. However, while thickening of the sarcolemma reminiscent of those at neuromuscular junctions are seen in aneural muscles, the synaptic folds that form in this situation are shallow and few.

Because extraocular muscles contain SIF and MIF types, the mechanisms of neuromuscular junction formation and stabilization must be different from those that are operative in other skeletal musculature. During development, initial synaptogenesis upon a putative skeletal muscle twitch fiber or extraocular SIF excludes the formation of junctions at other sites on that same fiber. The fiber may then be transiently polyneuronally innervated, but the multiple contacts are restricted to the one synaptic site. The stacking of axon terminals at individual neuromuscular junctions of E135 monkeys may represent such transient multiple innervation of SIFs. In contrast, synaptogenesis must be allowed to occur at numerous sites along the length of individual MIFs. Several muscle extraocular matrix and cell surface markers (see Sanes for review) have been hypothesized to serve as trophic factors that attract ingrowing axons. The restricted expression of such markers to the neuromuscular junction may be a consequence of the myonuclear aggregations seen in SIFs at E135. Perhaps differences in the pattern of expression of such markers in extraocular muscles are responsible for the diverse innervation pattern seen in these muscles. Furthermore, at all stages examined in the present study, there was a surprising paucity of axonal growth cones, suggesting that the remodelling of neuromuscular junctions is minor or that it must occur rapidly enough to have been missed in the present study. It has been established that skeletal muscle fibers initially are innervated by multiple axons and that all but one retract during the perinatal period. The timing and duration of transient multiple innervation of SIFs in the extraocular muscles are unclear. Oculomotor motoneuron death is observed in the perinatal period, however, suggesting that the competition for synaptic sites in extraocular muscle is similar to that seen in other skeletal muscles.

Functional Considerations

The basic determination of general fiber types (ie, slow versus fast) is evident early in development as the definitive ATPase staining pattern in cat extraocular muscles is observed at birth and the relative proportions of fiber types do not change thereafter. Mitochondrial pattern and microvascular network, however, were the last features to fully develop, occurring predominantly in the postnatal period for rat, cat, and monkey. These features are responsible for determining the aerobic energy production capacity of muscle fiber and thereby translate directly into the relative fatigability of the fiber. In the cat, motor unit studies show that slow fiber systems are mature by 6 wk postnatal, but fast systems continue to develop until the adult stage. These findings are consistent with pres-
ent data that indicate early maturation of global MIFs and significant postnatal increases in fiber diameter and mitochondrial content. Convincing evidence exists that fatigue resistance is shaped by the functional demands placed upon the muscles by developing visual and oculomotor systems. Hanson and coworkers have shown that the differentiation of succinic dehydrogenase (a mitochondrial oxidative enzyme) staining patterns that lead to definitive extraocular muscle fiber types in cat begins as late as P28. In addition, when normal visual system maturation is impaired by monocular lid suture in normal cats or impaired genetically in albino Siamese cats, the extraocular muscles exhibit slower contraction times and reduced fatigue resistance. The anatomical correlate of these functional changes was reduced fiber diameter and lower capillary density/fiber. The development of motoneurons subserving eye movement function also is impaired by monocular lid suture. Interestingly, the morphological changes induced by visual deprivation did not alter the overall rotational stiffness of the eye in the orbit.

In summary, extraocular muscles exhibit greater structural and functional diversity than any other skeletal muscle. This muscle complexity reflects the diversity and plasticity inherent in eye movement control systems. We interpret the pattern of extraocular muscle development to indicate an adaptation to the changing demands placed upon these muscles. The sequential development of the six muscle fiber types may reflect the functional pressures the extraocular muscles are exposed to by maturing visual and visuomotor systems. Several questions remain regarding extraocular muscle development. First, whether there is any difference in the fiber types derived from the primary and secondary generation myoblasts formed in these muscles is undetermined. Second, the role of intrinsic muscle properties in generation of the unique extraocular fiber types and the stage or stages at which neural influences may play a role in such determinations remain to be established. Finally, also unexplored are the positional or neural cues responsible for the formation of the orbital and global muscle layers in extraocular muscle.

Key words: extraocular muscle, myogenesis, eye movement, neuromuscular, monkey.

Acknowledgments

The authors thank Dr. Anita Hendrickson for providing the fetal monkey extraocular muscles for these studies. The technical expertise of Linda Simmerman and comments on an earlier version of the manuscript by Drs. Philip Bonner and Robert Spencer also are much appreciated.

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