Development of homogeneous fast and slow motor units in the neonatal mouse soleus muscle

TORMOD FLADBY and JAN K. S. JANSEN
Institute of Physiology, University of Oslo, Karl Johansgate 47, N-0162 Oslo 1, Norway

Summary

We studied the fiber type composition and contractile properties of mouse soleus motor units at 2 days, 5 days and 2 weeks of age. We used Lucifer Yellow injection to mark muscle fibers belonging to the same motor unit in the two youngest age groups, and the traditional method of glycogen depletion in the oldest. The age groups were chosen because 2 days is at the end of muscle fiber production; 5 days is at the start of synapse elimination in the muscle and 2 weeks is at the end. Muscle fibers were classified as fast (F) or slow (S) on the basis of their myosin heavy chain (MHC) content, as determined by different monoclonal antibodies.

Motor units are already dominated by either F- or S-fibers at 2 days, suggesting an early preferential innervation of the two types of fibers. A substantial part of the remaining refinement of the innervation takes place during the next 3 days, while the total number of terminals in the muscle remains constant. This is most easily explained by an exchange of aberrant for correct synapses during this period. A smaller part of the refinement of the innervation occurs during the subsequent period of synapse elimination.

Key words: neuromuscular junction, selective innervation, exchange of nerve terminals.

Introduction

One of the fundamental questions in neural development is how neurons connect to correct target cells. A set of correct connections is the end result of axonal and target tissue interaction that is initiated in embryonic life. In mouse hindlimbs, motor axons are guided to the correct muscles which they reach after muscle cleavage at embryonic day (E) 14. The next set of decisions concerns the formation of contacts within the muscle, between correct pre- and postsynaptic cell types. A reliable identification of cell types obviously is a prerequisite for the study of type-specific synapse formation during development.

Skeletal muscle fibers are subdivided in two main groups, fast (F) and slow (S), based on covariation of electrophysiological, contractile, metabolic and molecular properties. Of these, the myosin heavy chain (MHC) composition is the most frequently used for the classification of muscle fibers. In the adult, the F- and S-muscle fibers are collected in homogeneous motor units innervated respectively by fast and slow motoneurons (Burke, 1981).

As far as we know, all the future innervating axons are present in the muscle shortly after the first contact has been made (Landmesser, 1980). The muscle, however, is immature. Myogenesis appears to occur in early (until E 14) and late (after E 16) phases, with a lag phase in between (Ontell and Kozeka, 1984a,b). These phases give rise to primary and secondary myotubes, which later tend to give rise to S- and F-muscle fibers, respectively (Narusawa et al. 1987). The former express both embryonic (E) MHC, and S-MHC. The latter initially express both E- and neonatal (N) MHC. Some fibers of either type are probably transformed to the other, apparently independently of innervation (Condon et al. 1990b). In the mouse soleus, the expression of E-MHC starts to decline early in the 2nd postnatal week. The substitution of N- with adult F-MHC takes place towards the end of this week.

To study how motor neurons selectively innervate either of these fiber types, the development of fast and slow motor units in terms of fiber type composition and twitch-time to peak was studied at 2 days, 5 days and 14–17 days of age. These ages were chosen because 2 days is at the end of muscle fiber production in the mouse soleus muscle, 5 days is at the starting point of synapse elimination and 14 days is at the end of synapse elimination. Between 2 and 5 days, there is a steady, high level of hyperinnervation of the muscle. Between 5 and 14 days, the number of synaptic contacts in the muscle is reduced from about 4000 to about 700 (Fladby, 1987). The experiments were thus designed to study the precision of the innervation pattern at an early stage and its change during the neonatal period.

Depending on age, either of two methods was employed to study the fiber type composition of motor units. Lucifer Yellow (LY) injection was chosen for the
two youngest age groups. At these ages, the average motor unit constitutes about one third of the muscle. Fibers belonging to one motor unit are thus easy to find, and fibers with an endplate potential (epp) from an isolated motor axon were injected with the dye. Many of the synaptic terminals in these muscles are too weak to activate their fibers. Nerve stimulation cannot deplete these fibers of glycogen. Therefore the injection method is more sensitive in young muscles. For the oldest age group, the situation is the reverse, and glycogen depletion is the method of choice. Muscle fiber types were subsequently determined by antibodies to myosin heavy chains (MHCs). We found that the motor units were dominated by either fast or slow fibers already at 2 days, and that most of the remaining refinement occurs before net synapse elimination which starts at 5 days. A smaller additional refinement takes place during the subsequent period of net synapse elimination, until 2 weeks of age.

Materials and methods

Preparation and tension recordings

Soleus muscles (NMRI strain) from mice age 2-17 days were dissected with the proximal end of the fibular bone and nerve supply including L4 and 5 ventral roots. Superfusion apparatus and tension recording techniques have been described (Fladby, 1987). For the isolation of single motor axons, the ventral roots were carefully separated into fine filaments. By increasing stimulus intensity to at least 5 times the threshold for the isolated axon, the frequency of stimulation required for fused tetanic contraction for the muscle units were then recorded. The subsequent procedure differed for the 2 and 5 day muscles, which were injected with dye through microelectrodes, and the 2 week old muscles, which were glycogen depleted.

Glycogen depletion

The procedure is similar to that used by Thompson et al. 1984. After isolation of a single soleus axon, the frequency of stimulation required for fused tetanic contraction for the motor unit was determined (normally 40-70 Hz). The motor unit was then stimulated with 0.8 s trains of that frequency every second, until complete fatigue. During this procedure, the superfusion fluid was gassed with nitrogen. Then tension was allowed to recover, while stimulating at 1 Hz with single shocks and superfusing with oxygenated fluid. Such a cycle normally lasted about 15 min, and was repeated 6 times or more, until the tension no longer recovered. The contralateral muscle was kept in the same bath without stimulation as control.

Intracellular recordings and injections

Intracellular recordings were made with microelectrodes filled with 0.1 M LiCl, 3-5 mM Lucifer Yellow CH dye (LY), resistance>100 megohms. To prevent contractions, both ends of the muscle were crushed midway between the tendon and the end plate region (Barstad, 1962). While the isolated axon was stimulated, eps were recorded in the muscle. Eps were identified as unitary potentials with fast (3-5 ms) rise time and definite quantal variations. In this way, signals due to artefactual or natural junctions between fibers were avoided (Schmalbruch, 1982; Soha et al. 1987). Fulfilling these criteria, the fibers were subsequently electrophoretically injected with LY dye. Both dorsal and ventral surfaces were systematically sampled. Muscle fibers were injected with 1 Hz pulses of 0.5 s 2-5 nA currents, for 5-10 min each. The stability of the penetration was assured by a stable membrane potential, and the continued presence of the epp through the injection period. After about 10 fibers in each motor unit had been filled with the dye, the acute experiments were terminated. Adequate filling of surface fibers was determined by fluorescent microscopy at this stage. This is illustrated in Fig. 1, which is a randomly injected 5 day muscle. The spacing of the fibers is of course not typical for the distribution of fibers in a motor unit.

Histology and immunohistochemistry

After the acute experiments, the muscles were fixed in 1 % paraformaldehyde for 5 min, and then kept for 1 h in 5 % dimethylsulphoxide/20 % sucrose/0.1 M phosphate buffer pH 7.4 for cryoprotection. They were packed in fixed liver tissue (3.5 % paraformaldehyde) and frozen in isopentane cooled with liquid nitrogen. They were subsequently transversely sectioned at 5-10×10^-6 M thickness in a cryostat (-25°C). Four neighbouring sections from different levels, spaced 0.1 mm apart were mounted on slides. Alternate sections were incubated with antibodies against F- and S-myosins overnight at +4°C. The slides were then

![Fig. 1. Lucifer Yellow labeled fibers. The dye has been injected in randomly selected surface muscle fibers. Whole mount view of 5 day old muscle which had been crushed (arrow) at one end. Injection sites can be identified by a central thickening in some fibers. The dye diffused rapidly throughout the undamaged part of the fibers. A few fibers are weakly labeled beyond the crush. We saw no sign of dye leaking from the injected fiber to its neighbours. Bar, 1 mm.](image-url)
thoroughly washed (4h, 4°C) with 0.2% bovine serum albumin in phosphate-buffered saline prior to incubation with the secondary antibodies. The sections were washed again, mounted in polyvinyl alcohol and viewed in a fluorescent microscope. The initial fixation was required to retain the LY in the fibers. However, the fixation was minimal since the fixed muscles were more inclined to disruption during freezing. Sections incubated without primary antibodies were inspected as controls for unspecific labelling. From glycogen-depleted muscles and control muscles, alternate sections were also stained for glycogen with the PAS/Schiff method (Sigma). Section thickness here was regularly 15 x 10^{-6} m. The fibers counted as depleted were distinctly paler than any control fibers. Using this procedure, from 50 to 80% of the fibers in a motor unit were included, based on the size estimated from its twitch tension. The inability to deplete all the fibers of a motor unit is a common experience in attempts to deplete muscle fibers of glycogen in in vitro preparations (Thompson et al. 1984; Jones et al. 1987a). All sections were scored independently by the two authors.

Antibodies

Two different monoclonal primary antibodies were used for the routine classification of muscle fibers. One (BAD) was specific for slow (S)-MHC at all stages. The other (BF 13) was specific for adult fast (F)-MHC and for neonatal (N)-MHC. Both antibodies were generous gifts from Dr S. Schiaffino, Padova. We also used two other antibodies (N2-221 and N1-551) against N-MHC and adult F-MHC. These stained like BF 13. Furthermore, we used an antibody (N 2.696) selective for type 2A-MHC, and one (F 1.652) selective for E-MHC. These were supplied by Dr W. J. Thompson, Austin and Dr Helen Blau, Stanford. As secondary antibodies, we used a commercially available rhodamine-conjugated polyclonal rabbit anti-mouse serum (Boehringer Mannheim).

Control for sampling errors

In the mouse soleus muscle, there are about equally many fibers of each type, and they are evenly distributed over the cross-section of the muscle (Vaughan, 1974). As mentioned above, fast type fibers are younger, and tend to have a smaller caliber in young muscles. As fibers are sampled by random poking with a microelectrode, this will introduce a sampling bias in favor of slow fibers. The magnitude of this bias can be closely approximated by the ratio of the diameters of fast and slow fibers, multiplied by the number of fibers of each type. In a 2 day muscle, we found a bias in favor of S-fibers of 0.56. Correcting the fiber type distributions in Fig. 3 with this factor changed the distribution insignificantly and was disregarded. Areas and fiber counts in this muscle were automated with an image analysis system (Zeus, Pixelwerks, Bergen). In two other 2 day muscles, 233 random fibers were manually measured and the corresponding biases in favor of S-fibers 0.58 and 0.54. At 5 days the difference between F- and S-fibers is marginally smaller. Random LY injection of fibers in a 5 day muscle gave 10 F-fibers, 12 S-fibers and 3 double staining fibers (see Results), this gives $P=0.54$ for hitting slow fibers. Manual measuring of 214 fibers from two 5 day muscles gave a similar $P=0.55$.

Results

Muscle fiber types

Two days after birth, most mouse soleus muscle fibers could be classified as belonging to one of two different types based on their myosin content. About 55 percent of the fibers in a cross-section of the muscle were labeled with the antibody to slow MHC (BAD 5) and therefore classified as S-fibers. These fibers were also weakly labeled by BF 13, the antibody reactive to F- and N-MHC (Fig. 2B,C). Virtually all the remaining fibers were strongly labeled by BF 13 and not by BAD 5 and were therefore classified as F-fibers.

It was surprising that BF 13, the antibody to N- and F-MHC, consistently labeled S-MHC-positive fibers above background (Figs 2, 5). This might be due to either a cross-reaction with E-MHC, which is still present in all fibers at day 5 (our observations, not shown), or to the expression of small amounts of N-MHC also in the S-fibers. To clarify this, we tried two additional antibodies (N 2-221 and N 1-551) against both N- and F-MHC. These two antibodies do not react with E-MHC or with S-fibers in the prenatal rat soleus in the muscle (Condon et al. 1990a). However, they labeled the S-fibers of the 2 and 5 day mouse soleus as our original BF 13 antibody. Hence, we suggest that the S-fibers of the newborn mouse soleus, unlike the rat soleus, express a small amount of N-like MHC at 2 and 5 days.

A small proportion of the muscle fibers (about 5 percent in a cross-section) reacted strongly to both BAD 5 and BF 13. These are probably what has been described as transforming fibers in the prenatal rat soleus (Condon et al. 1990a). In the mouse soleus, they were present at both 2 and 5 days (Figs 2, 5). Fibers of this type either showed equally strong labelling for S- and N-MHC at all longitudinal levels examined (FS-fibers) or they had segments dominated by either S- or N-MHC. The latter type of fiber had a ‘dominant’ phenotype and was classified and included in the material accordingly as S- or F-fibers. The SF-fibers, which had equally strong labelling at all levels examined, (2 in the 2 day and 3 in the 5 day material) were excluded from the material. This did not make a significant difference to the distribution of fibers presented in Fig. 3.

Among the 8 transforming fibers in the 2 day material, two fibers strongly expressed both types of myosin at all levels examined. Of the other 6, 3 fibers continuously expressed S-MHC and, in one region, also N-MHC. The remaining three fibers showed equally strong labelling at all levels examined, (2 in the 2 day and 3 in the 5 day material) were excluded from the material. This did not make a significant difference to the distribution of fibers presented in Fig. 3.

Among the 8 transforming fibers in the 2 day material, two fibers strongly expressed both types of myosin at all levels examined. Of the other 6, 3 fibers continuously expressed S-MHC and, in one region, also N-MHC. The remaining three fibers showed the opposite pattern. Of 15 transforming fibers in the 5 day material, 3 expressed both types of myosin at all levels. One of these was examined at 10 levels evenly spaced through the muscle, the other 2 only at two levels. Nine fibers continuously expressed S-MHC, and in one region, also N-MHC. The remaining 3 fibers showed the opposite pattern.

We conclude from this that the majority of muscle fibers in the newborn mouse soleus can be classified from their MHC content into two groups, which probably represent the prospective fast and slow fibers of the mature muscle.

2 day motor units

Stimulation of single soleus motor axons in ventral root
filaments gave information on twitch time to peak and amplitude of contraction for individual motor units. While the twitch contraction times vary over an appreciable range, there was no significant correlation between contraction time and twitch amplitude. This suggests that different types of motor units in the muscle are fairly equal in size at this stage. This was the case for the 5 and 14–17 day materials as well.

A sample of muscle fibers from each motor unit was identified by microelectrode recording of unitary epps during stimulation of its motor axon. Such fibers were iontophoretically labeled with Lucifer Yellow for subsequent immunohistochemical classification of the fibers (Fig. 2B,C). 8 to 13 fibers (average 10.2) were identified in each of 10 different motor units. Of these 102 fibers, 64 were classified as S-fibers and 36 fibers as F-fibers according to the criteria defined in the previous section.

Composition of motor units

Most of the motor units examined at 2 days contained both S- and F-fibers among the Lucifer-labeled sample. To illustrate the composition of the motor units, they were ranked according to the proportion of F-fibers in the sample and their distribution is illustrated in Fig. 3A. It F- and S-fibers were randomly innervated

![Fig. 2. LY injected muscle fibers. 2 day motor unit. (A) Two injected muscle fibers viewed with LY filters. (B) Same section viewed with rhodamine filters, which reveal reactivity to S-MHC (antibody BAD-5). Large arrows to LY-labeled fibers. The one to the left is S-MHC positive. (C) Neighbouring section. Reactivity to N-MHC (antibody BF 13). Large arrows indicate the two LY-labeled fibers. The fiber at left is classified as S-fiber, the fiber at right as F. Section C is closer to the injection site of the F-fiber and it is swollen. There were 10 additional LY-labeled fibers from this motor unit (3 S, 7 F). Short arrow indicates a FS-fiber (see text) that had not been LY injected.

![Fig. 3. Fiber type composition of motor units. Fraction of muscle fibers in each motor unit with an F-staining pattern (individual fibers that are negative for S-MHC, strongly positive for N/F-MHC) along the x-axis, number of units at each fraction along the y-axis. (A) 2 days, 10 motor units with an average of 10.2 (range 8–13) fibers labeled in each. (B) 5 days, 18 motor units with an average of 10.8 (range 7–14) fibers labeled in each. (C) 2 weeks, 7 motor units with an average of 21.9 (range 16–38) labeled in each. The histograms show an increasing specificity of innervation during the neonatal period.](image-url)
the distribution of units in the diagram would be unimodal. In contrast, the observed distribution is bimodal. A significant difference from random innervation was found using a χ² test (χ²=29.12, P<0.01, df=9). This suggests that the individual motor axons preferentially innervate either F- or S-muscle fibers already by postnatal (P) day 2.

In Fig. 3 the 'transforming' fibers (see above) have been included according to their dominant phenotype. In contrast to other limb muscles, a particularly large fraction of the secondary generation myotubes in the soleus differentiates to S-fibers (Condon et al. 1990a). It might be that all myofibers expressing S-MHC should be classified as prospective S-fibers. This changes the distribution of the motor units in Fig. 3A in the direction of more heterogeneous motor units. The distribution was still bimodal and significantly different from random (χ²=26.73, P<0.01, df=9). However, it cannot be excluded that S-MHC is transiently expressed in some prospective F-muscle fibers (Dhoot, 1986). Furthermore, the frequency of F- and S-fibers was similar at 2 and 5 days and the classification according to dominant phenotype improved the correlation between fiber composition and twitch contraction time of the motor units (Fig. 4). We therefore retained this classification in the presentation of the distributions. The distribution of the 5 day motor units (Fig. 3B) was not significantly changed by classifying all the transforming fibers as prospective S-fibers.

**Speed of contraction**

The contractile properties of mature motor units are related to fiber type. Their twitch time to peak is shorter for F- than for S-motor units and bimodally distributed in the mature mouse soleus (Fladby, unpublished). At 2 days of age the motor unit twitch times to peak ranged from 73 to 100 ms, and was unimodally distributed. However, when related to the composition of the motor units, the twitch time to peak decreased with increasing fraction of F-fibers (Fig. 4 (o)). The linear correlation between the two is significantly different from zero (r=0.86, P<0.05). This suggests that the observed bimodal distribution of motor unit composition is genuine rather than due to misleading sampling in the newborn muscles. Whether the apparently continuous distribution of motor unit twitch times is due to variations in fiber type composition within F- and S-motor units or due to a higher proportion of immature fibers in some motor units is still unresolved. Presumably both factors contribute.

**5 day motor units**

The same procedure was followed to study the muscle at 5 days. At this time the muscle fibers were larger and the expression of myosin isozymes was more distinct. 195 muscle fibers in 18 different motor units were labeled with Lucifer Yellow. Among these, 114 fibers were immunologically classified as S- and 78 as F-fibers. Three FS-fibers were excluded from the material used to characterize the fiber content of the individual units.

**Composition of motor units**

Fig. 3B illustrates the distribution of motor units ranked according to their fraction of F-fibers. At this age, the distribution of fibers was significantly more segregated than at 2 days (one-sided Wilcoxon rank-order test, with lowest ranks allocated to the most homogeneous motor units, P<0.02) indicating that the younger motor units were more heterogeneous in terms of fiber types. An example of labeled fibers from a motor unit and their identification with MHC antibodies is shown in Fig. 5.

**Speed of contraction**

The twitch time to peak of the 5 day motor units is given according to their fraction of F-fibers in Fig. 4 (+). As for the 2 day material the contraction time is reduced with increasing content of F-fibers (r=0.88). Moreover, as for the composition of the motor units, there is significantly less scatter in the contraction times in 5 day material (P<0.02, Wilcoxon rank-order test, one sided) consistent with the greater homogeneity of the motor units in the more mature muscles.

**14-17 day motor units**

At this age, the size of the motor units in the mouse soleus is reduced by more than 80 per cent, and virtually all the muscle fibers are singly innervated (Fladby, 1987). Hence, the fibers are difficult to find with a microelectrode and we preferred the glycogen depletion technique (Edström and Kugelberg, 1968) to determine the composition of the motor units. 7 motor units were glycogen depleted following a protocol.

---

**Fig. 4.** Contraction times and composition of motor units. Fraction F-fibers (as in Fig. 3) and normalized twitch time to peak for the motor units in Fig. 3. Motor unit twitch time to peak expressed as percentage of whole muscle time to peak. (o) 2 day motor units, (+) 5 day units, (D) 2 week units. The absolute values of the twitch contraction times varied appreciably, partly because the measurements were performed at room temperature (21 to 24°C) and probably also on account of differences in maturity between mice of nominally the same age. For the 2 day muscle twitch contraction times ranged from 95 to 140 ms (mean 103 ms), for the 5 day muscles the corresponding values were 75 to 130 ms (mean 91 ms) and for the 14 to 17 day muscle the values were 46 to 78 ms (mean 64 ms).

---
Fig. 5. LY-injected fibers. 5 day motor unit. 5 injected fibers (arrows) viewed with LY fibers in two neighbouring sections (A1,B1). Below, same sections viewed with rhodamine filter to show reactivity to S-MHC (A2) and F-HMC (B2). The deepest of the LY-labeled fibers is a ‘transforming’ FS-fiber. The fiber to the left is a F-fiber. The 3 remaining labeled fibers are S-fibers. Six additional fibers were LY-labeled in this unit, all were S-fibers. Reproduced in black and white, the distinctness of the LY signal in A1 and B1 has suffered since our LY filter did not completely exclude the strong rhodamine signal from heavily labeled fibers.

introduced by Thompson et al. (1984). An example of cross-sections from one such muscle is given in Fig. 6. 16 to 38 muscle fibers were unambiguously glycogen depleted in the muscles. This is less (by about 40 percent on the average) than the number of fibers in the units as estimated from their tetanic contractile force. The incomplete depletion of the activated motor units is due to the in vitro procedure. To prevent unspecific depletion, the anoxic periods had to be minimized.

At this juvenile stage, the immunological staining properties of the fibers had changed. Staining for F-MHC and S-MHC were mutually exclusive and we found no fibers reactive to both antibodies.

Composition of motor units

Ranked according to fiber composition, the motor units were sharply segregated in two groups (Fig. 3C). Similarly the twitch contraction times were distinctly segregated for the two types of motor units (Fig. 4). Yet the motor units were not yet completely fiber type homogeneous. Five depleted fibers with an aberrant phenotype were found in the samples from the 7 motor units. Four of these were S-fibers in F-motor units and the last was a F-fiber in a S-motor unit. Since our motor units were incompletely depleted, this corresponds approximately to one mismatched fiber per motor unit in the muscle.

Discussion

The main message of the present findings is the demonstration of a progressively increasing functional homogeneity of the composition of motor units over the first 3 neonatal weeks. From earlier work, the differentiation of the skeletal muscle fibers, as well as the perinatal reorganization of the pattern of innervation in rodents is known in considerable detail (Narusawa et al. 1987; Condon et al. 1990a; Slater, 1982; Fladby, 1987).

Drawing on this and related information, it is possible to suggest a plausible scenario for some of the major developmental events contributing to the establishment of the pattern of innervation of the soleus muscle over the perinatal and early postnatal period. The available information has been obtained partly in rats and partly in mice. However, qualitatively the neuromuscular development is very similar in the two species, and we
believe that inferences based on comparison between the two are acceptably safe.

The perinatal patterns of innervation

The mouse soleus, along with other shank muscles, is first innervated on E 14, soon after the arrival of the motor axons at their target. At this time, only the primary generation of myotubes have been born and they represent approximately 15% of the final complement of fibers in the rat soleus (Narusawa et al. 1987; Condon et al. 1990a), which would mean about 100 fibers in the mouse soleus. While expressing only E-MHC initially, all these fibers soon start to produce S-MHC in addition, and they all remain as slow fibers in the mature muscle.

These fibers are the only targets for the motor axons when the muscle is first innervated. In the chick, the full complement of motor axons is present in the muscle when the first innervation takes place (Landmesser, 1978). Thus, initially the 20 motor axons of the mouse soleus presumably share the innervation of the available primary muscle fibers. This implies that the prospective slow and fast motoneurons at first innervate only future slow muscle fibers. As fast motoneurons innervate some S-muscle fibers even postnatally, it is probably safe to assume that they do so also before the F-fibers are produced.

Fig. 6. Glycogen depleted fibers. 16 day motor unit.
(A) PAS/Schiff-stained section. Circles indicate 7 fibers depleted of glycogen.
(B) Neighbouring section, rhodamine filter, primary antibody to S-MHC (BAD 5). The depleted fibers (o) all stain for S-MHC. (C) Neighbouring section, rhodamine filter, primary antibodies to F/N-MHC (BF 13). The depleted fibers (o) are not labeled with this antibody. All the 16 fibers depleted in this muscle were S-fibers.
After a delay of a day or two myogenesis is resumed by the generation of secondary myotubes (Condon et al. 1990; Ontell and Kozeika, 1984a,b). In the mouse soleus, this goes on until P 2 when the full complement of about 700 muscle fibres is established. Of these, close to 600 are derived from the secondary generation. These secondary fibers all initially express N-MHC and only a small proportion of them in addition express S-MHC during embryonic development (Condon et al. 1990a). Over the first two postnatal days an increasing number of the secondary generation fibers express the S-MHC at the expense of their content of N-MHC, so that by P day 2 the pattern of fiber differentiation closely resembles that of early mature muscles. Just over 50% of the fibers contains S-MHC and a small amount of N-MHC while the remaining fibers strongly express the N-MHC, and the two types of fiber are evenly distributed over the cross-section of the muscle.

At this time, the motor units have reached their maximal extent and all the fibers are heavily polyinnervated (Fladby, 1987). There are about equal numbers of fast and slow motor units, and the two types are of comparable size. As presently shown, the motor units, while dominated by either F- or S-fibers, contain a significant component of inappropriate fibers. For the fast motor units, the fraction of S-fibers is as high as 1/3. With half of the total 4200 terminals in the muscle belonging to F-motor neurons this means that approximately 700 F-terinals innervate S-muscle fibers. By day 2 the muscle contains about 350 S-muscle fibers of which 100 belong to the original group of primary myotubes (Narusawa et al. 1987). It is unlikely that the 700 F-terinals on S-fibers simply represent the retention of the F-terinals on the primary fibers. Most likely they are distributed to S-fibers from both the primary and secondary generation of myotubes.

The slow motor units are more homogeneous than the fast at this age and contain only about half as many inappropriate fibers. These are secondary myofibers expressing only the N-MHC. Hence, during the dramatic expansion of the target muscle during secondary myogenesis the fast and slow motor neurons both contribute to the innervation of the new targets. By P 2 the fast motoneurons have done about four times better than the slow in innervating the newly generated F-fibers. The slow motor neurons have had a comparable preference for innervating the S-fibers of the secondary generation, while retaining probably most of the primary generation myotubes.

From P 2 till P 5, the total number of motor nerve terminals (Fladby, 1987) and the number and types of muscle fibers remains essentially constant. Yet, the present observations show that the frequencies of discordant muscle fibers in F- and S-motor units are significantly reduced, while the size of the motor units remain essentially unchanged. Hence, the increased selectivity in the innervation of the muscle is probably due to exchange of terminals from inappropriate to appropriate muscle fibers. Presumably fast motor neurons tend to lose their original terminals on the primary fibers and the secondary S-fibers while compensating this loss by making new terminals on the secondary F-fibers. The slow group of motor neurons similarly lose terminals on the secondary F-fibers while establishing new contacts with the secondary S-fibers. The rate of loss of mismatched terminals is more than two times higher for the fast than for the slow motor units over the terminal exchange period.

The exchange of terminals can be understood in terms of a competitive interaction between terminals innervating the same muscle fiber. Such a competitive process is well established for muscle fiber innervation (see Jansen and Fladby, 1990 for review). Two classes of factors are important for the outcome of the competition. The one is determined locally at the endplate and is related to factors such as efficiency of uptake of a trophic substance and how well the interacting terminals are 'matched' to different functional types of muscle fibers. The other class is related to the global properties of each motoneuron, most directly to the number of terminal branches each can maintain. The extent of terminal branching is inversely related to the 'competitive vigor' of the motoneuron. Hence, as a motor neuron loses a terminal on an inappropriate muscle fiber on account of unfavourable matching to its surface membrane, it will at the same time be competitively favoured to maintain its appropriate terminals and more inclined to generate new terminals, preferably on correct fibers.

Applied to the early innervation of the mouse soleus, this implies that the fast motor neurons will have a competitive advantage for the innervation of the newly formed N-MHC containing myotubes at the initiation of the secondary myogenesis. As the competitive vigor of the fast motoneurons is progressively reduced the secondary N-MHC containing myotubes also with a fast phenotype are increasingly incorporated into the expanding slow motor units. On this view, the differential timing of the production of F- and S-muscle fibers during myogenesis is one of the factors that may explain the heterogeneity of the early motor units.

This scenario postulates a recognition mechanism between nerve terminals and postsynaptic membrane presumably based on particular membrane epitopes, which must be different for the two classes of motoneurons and muscle fibers. The relevant epitopes determine the degree of matching between the synaptically related membranes. The specificity of the mechanism can not be absolute, since S-motoneurons do not strictly innervate S-fibers and F-motor neurons do not strictly innervate F-fibers. Hence, all fibers are innervated and all terminals have targets even in the immature muscle. In this argument, we presume that the membrane epitopes and the MHC antigens are expressed from the initial formation of the myotubes. However, an additional contribution to the early mismatching from a moderate delay in the development of the target recognition mechanism has not been excluded.

The final stage of the perinatal reorganization of muscle innervation lasts from P 5 to 15 in the mouse soleus, and is characterized by a continuous net loss of nerve terminals from the muscle until each fiber is
innervated only by a single terminal. As during the terminal exchange period, the rate of loss of mismatched terminals is higher for fast than for slow motor units. A similar difference has been reported for the rabbit soleus (Callaway et al. 1989). The total number of terminals is reduced by a factor of six (Fladby, 1987) and, among the 3500 terminals that are lost, close to 500 are mismatched terminals. If the loss of terminals had been evenly distributed among appropriate and inappropriate terminals, the loss of inappropriate terminals would have been significantly smaller, just over 400. Hence, the correctly connected terminals are competitively favoured. Even though the preference for correct terminals is not very strong, the frequency of inappropriate fibers in the mature motor units would have been about 4 times higher without it.

A diagrammatic summary of this scenario is given in Fig. 7. Although hypothetical, the prenatal part shows that the future fast motoneurons preferentially establish new connections with the newly generated future F-muscle fibers while the slow motor neurons have the future S-muscle fibers as their preferred targets. This happens at a stage where our insight into the differentiation of the two classes of motoneurons is fragmentary, but it is hard to see how it could take place without specific recognition mechanisms in the two types of neuron as early as this stage. This is supported by the selective reinnervation of muscle at slightly later stages (Soileau et al. 1987) and by the later differentiation of motoneurons with distinct functional properties (Navarette et al. 1988; Fulton and Walton, 1986).

Perinatal events in other muscles

The generation of homogeneous F- and S-motor units has also been studied in the rat soleus (Thompson et al. 1984) and the rat lumbrical muscle (Jones et al. 1987a).

![Fig. 7. The development of homogeneous fast and slow motor units. Average fraction of S-fibers in F-dominated (C) and S-dominated motor units (●) is plotted against time. Bars give s.d. Fully drawn lines illustrate the observed postnatal development, stippled lines the suggested prenatal development. Selective innervation is already present at 2 days. Most of the remaining refinement takes place during the following three days, before the ensuing period of net synapse elimination in this muscle. The timing of secondary myogenesis is arbitrarily taken to be equal to that of the mouse EDL (Ontell and Kozeka, 1984a).]

As mentioned the soleus muscle of rat and mouse have very similar developmental histories, and the results from the rat are easily incorporated into the present picture based on mice. Thompson et al. (1984) used glycogen depletion to identify muscle fibers belonging to individual motor units. Hence, P8 muscles were their youngest age group. For these they found motor units dominated by either F- or S-fibers while still containing some inappropriate fibers, not unlike the present finding in mice at 5 days. However, in the rat, the S-motor units were less homogeneous than the F-motor units both at 8 days, and to a much smaller extent at 16 days. The situation was the opposite in the mouse at 5 days. However, the discrepancy is small and probably no more than can be explained by the 3 day difference in age of the rat and mouse material, and the higher rate of loss of mismatched terminals in F- than in S-motor units. By 8 days of age, mouse soleus motor units may well have reached levels of homogeneity comparable to those of the rat. After the synapse elimination period, the distribution of inappropriate fibers is similar in the two materials.

The developmental history of the rat 4th lumbrical muscle is different from that of the soleus. Myogenesis continues for two weeks postnatally. More than two thirds of the fibers are generated after birth (Jones et al. 1987b). Some of its primary fibers develop into F-fibers (Condon et al. 1990a). The fiber composition of the muscle is strongly biased in favour of fast fibers and there is probably only a single slow motoneuron among the eleven motoneurons to the muscle (Jones et al. 1987a). Examined between P days 3 and 5 the fiber composition of its motor units closely reflects the fiber composition of the whole muscle and suggests a random innervation of the motor units (Jones et al. 1987a). However, any tendency to selective innervation is expected to be less pronounced on account of the immaturity and the skewed fiber type composition of the muscle. Hence, the situation is not necessarily very different from that in the soleus.

The differentiation of skeletal muscle

The present view of the establishment of fiber-type-specific innervation of skeletal muscle leans heavily on the autonomous differentiation of the muscle fibers. From their studies on the newborn rat lumbrical muscle, Jones et al. (1987b) reached a similar conclusion. The main evidence for autonomous fiber differentiation in mammalian muscle is the development of essentially the normal pattern of distribution of F- and S-muscle fibre in aneurogenic muscles (Condon et al. 1990b). This, however, does not rule out some neural effects on fiber differentiation. For instance, the aneurogenic muscles usually contain less than half the number of fibers in normal muscles. In some muscles, the primary myotubes fail to express S-myosin before they convert to F-fibers. Apparently some effect of the nerve is required for the transient expression of S-MHC in these fibers (Condon et al. 1990b).

Activity-dependent conversion of muscle fibers is a well-established phenomenon in mature muscle. Acti-
vating the soleus fibers with a fast pattern of activity results in its slow fibers adopting many of the properties of fast fibers including the expression of F-MHC (Gorza et al. 1988). In the mouse soleus at 15 days, virtually all the fibers of the muscle are singly innervated and the few inappropriate fibers of its motor units are good candidates for activity-dependent fiber conversion. At appreciably older stages, many of the fast rat soleus fibers convert to the slow phenotype. This seems to occur by the conversion of whole motor units and is probably activity dependent (Kugelberg, 1976). Furthermore, by the end of the 2nd postnatal week the N-MHC in prospective fast fibers is substituted by adult F-MHC. This is a reversible process, which is normally controlled by the innervation of the muscle fibers (Schiaffino et al. 1988, 1990).

To conclude, several factors contribute to the differentiation of muscle fibers and the generation of homogenous motor units. Before birth, the autonomous differentiation of muscle fibers is a major factor. Innervation and the resulting pattern of activity in the fibers are of increasing importance at later stages. The prospective fast and slow myofibers are preferentially but not exclusively innervated by homotypic motor nerve terminals. The preference in innervation is probably based on the recognition of specific membrane epitopes. Most of the aberrant terminals are exchanged for appropriate terminals while the total density of innervation remains constant. The final refinement of the innervation takes place during the following loss of terminals in the muscle.

We thank Håvard Tønnesen for invaluable assistance, Dr Joel Glover and Dr Terje Lømo for discussions and improving the manuscript. Dr Knut Liestøl advised us on the statistics. Dr Stefano Schiaffino, Dr Wesley Thompson and Dr Helen Blau generously supplied the MHC antibodies.

References


(Accepted 18 April 1990)