Formation of primary and secondary myotubes in rat lumbrical muscles

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Summary

Numbers of myoblasts, primary myotubes and secondary myotubes in developing rat embryo hindlimb IVth lumbrical muscles were counted at daily intervals up until the time of birth, using electron microscopy. Motoneurone death at the spinal cord level supplying the lumbricals was assessed by counting axons in the 4th lumbar ventral root. Death of the motoneurones that supply the intrinsic muscles of the hindfoot was monitored by comparing the timecourse of development of total muscle choline acetyltransferase activity in control embryos with that in embryos where motoneurone death was inhibited by chronic paralysis with TTX, and by counting axons in the mixed nerve trunks at the level of the ankle at daily intervals. Condensations of undifferentiated cells marking the site of formation of the muscle were seen on embryonic day 15 (E15). Primary myotubes began to appear on E16 and reached a stable number (102 ± 4) by E17. Secondary myotubes first appeared two days later, on E19, and numbered 280 at the time of birth (E22). The adult total of about 1000 muscle fibres, derived from both primary and secondary myotubes, was reached at postnatal day 7 (PN7) so considerable generation of secondary myotubes occurred after birth. There was a linear correlation between the number of undifferentiated mononucleate cells in a muscle and the rate of formation of secondary myotubes. The major period of motoneurone death in lumbar spinal cord was during E16–E17, when axon numbers in the L4 ventral root fell from 12000 to 4000, but a discontinuity in the curve of muscle ChAT activity versus time indicated that death in the lumbrical motor pool occurred during E17–E19, after all primary myotubes had formed and before generation of secondary myotubes began. We suggest that motoneurone death, by regulating the final size of the motoneurone pool, regulates the ratio of secondary to primary myotube numbers in a muscle.

Key words: skeletal muscle, embryonic development, myoblasts, primary myotubes, secondary myotubes, cell junctions, motoneurone death, rat embryo.

Introduction

This is the first of two papers concerned with the determination of skeletal muscle fibre numbers during embryonic development. It has previously been shown (Harris, 1981) that primary myotubes develop autonomously, but generation of secondary myotubes requires both the presence and the activity of motor innervation. Now it is of interest to ask whether innervation is regulatory or permissive for the formation of secondary myotubes. In this paper, we give a quantitative description of some aspects of development of the IVth lumbrical muscle in the rat hindlimb and of its innervation; in the next paper (Ross, Duxson & Harris, 1987), we describe the effects of partial reduction of the muscle’s innervation during development.

The major features of skeletal muscle formation are well known. Following the seminal work of Kelly & Zacks (1969a), several authors have confirmed the biphasic nature of muscle development (Ashmore, Robinson, Rattray & Doerr, 1972; Ontell & Dunn, 1978; Harris, 1981; Stickland, 1982; Ontell & Kozeka, 1984a,b). Mononucleate myoblasts fuse to form primary myotubes which extend from tendon to tendon of the embryonic muscle (Dennis, Ziskind-Conhaim & Harris, 1981; Ontell & Kozeka, 1984a), forming a cellular framework for the later generation of secondary myotubes. Secondary myotubes form near the midpoint of primary myotubes under the basal
lamina, resulting in clusters of muscle cells within a single basal lamina. The secondary myotubes then grow longitudinally, eventually connecting with the muscle tendon and separating from the primary myotube as they acquire their own basal lamina. Subsequently, secondary myotubes are difficult to distinguish ultrastructurally from primary myotubes. Here we give a quantitative description of the normal development of a particular muscle (the IVth lumbral in the rat hindfoot) and of the timing of motoneuron death in its motor pool, as a prelude to a study of the neural regulation of muscle development (Ross et al. 1987).

Materials and methods

Electron microscopy (EM)

We chose the lumbral muscle for this study because an entire cross section fits on a single electron microscope grid, allowing all the myogenic cells to be identified and counted. The motor innervation to the IVth lumbral, previously described by Betz, Caldwell & Ribchester (1979), leaves the spinal cord in the L4, L5 and L6 ventral roots.

All experiments were performed on White Wistar rats. Pregnancies were dated by the appearance of a copulatory plug, with 9 a.m. on the day the plug was found being E0. Embryos from at least three different litters were taken at daily intervals from E15 onwards; the numbers of muscles and ventral roots from which photomontages were prepared are given in Table 1. PN28 tissues came from three litters of control animals employed in another study, currently in progress. The mother was killed with a blow to the head, and the embryos quickly removed and placed on ice for anaesthesia. They were then perfused through the heart with heparinized saline followed by fixative, both at 4°C. They were then postfixed in osmium tetroxide and uranyl acetate, dehydrated and embedded in phosphate buffer with glucose added as above to match the animals of different ages. Ultrathin sections were in all allowing the best comparison of axon number between regions.

This was chosen as the most compact region of this nerve, allowing the best comparison of axon number between animals of different ages. Sections with light microscopy. Muscles were sectioned in the midbelly endplate-containing region. Ventral roots were cut 1–2 mm proximal to the dorsal root ganglion. In one embryo at each day from E16–E19, branches of the plantar nerve were visualized in a cross section of the distal hindlimb at the level of the malleolus. This was chosen as the most compact region of this nerve, allowing the best comparison of axon number between animals of different ages. Ultrathin sections were in all cases mounted on single-slot Formvar-coated copper grids, stained with uranyl acetate and lead citrate and then viewed with a Phillips 300 or 410 electron microscope. Photomontages of entire lumbral muscles, ventral roots and all branches of the plantar nerve were produced at final magnifications in the range ×6300–11 400. Cells in the cross sections were classified and counted as described in the Results.

A serial and semiserial EM study was also made of a single E21 IVth lumbral muscle in order to clarify some aspects of the muscle organization. Sectioning commenced in the midbelly region of the muscle and continued into the tendon, a total length of about 400 μm. If the position of the origin is designated as zero along the longitudinal axis of the muscle, regions from 0–10 μm, 10–160 μm and 220–400 μm were serially sectioned and all sections collected; in remaining regions ultrathin sections were collected at intervals of 2.5 μm.

Choline acetyltransferase assays

Choline acetyltransferase (ChAT) (acetyl-CoA:choline O-acetyltransferase, EC 2.3.1.6.) activity was assayed in homogenates of whole hindfoot as an index of nerve terminal numbers in the intrinsic muscles of the foot. Embryos were taken as described above, and hindfoot removed by section through the ankle joint and placed in 20 or 50 μl of homogenization buffer depending on the age of

| Table 1A. Experimental materials used for electron microscopic studies |
|--------------------------|--------------------------|--------------------------|--------------------------|
| Experimental treatment   | Embryonic age (days)     | Number of muscles examined | Number of photomontages  | Number of ventral roots examined |
| Controls                 | PN28                     | 6                         | 6                        | 1                          |
|                         | 22                       | 2                         | 2                        | 1                          |
|                         | 21                       | 12                        | 12                       | 7                          |
|                         | 20                       | 4                         | 4                        | 2                          |
|                         | 19                       | 9                         | 9                        | 4                          |
|                         | 18                       | 4                         | 4                        | 3                          |
|                         | 17                       | 5                         | 5                        | 3                          |
|                         | 16                       | 3                         | 3                        | 4                          |
|                         | 15                       | 3                         | 3                        | 2                          |

| Table 1B. Experimental materials used for choline acetyltransferase assays |
|--------------------------|--------------------------|--------------------------|
| Experimental treatment   | Embryonic age (days)     | Number of muscles assayed |
| Controls                 | 21                       | 96                       |
|                         | 20                       | 32                       |
|                         | 19                       | 84                       |
|                         | 18                       | 58                       |
|                         | 17                       | 45                       |
|                         | 16                       | 23                       |
|                         | 15                       | 26                       |
| TTX                      | 15–21                    | 28                       |
|                         | 15–19                    | 6                        |
|                         | 15–17                    | 6                        |
the embryo, quick frozen in liquid nitrogen and stored at
−80°C until they were assayed. Assays were made with the
technique of Fonnum (1969), using [14C]acetyle CoA (Amer-
sham), specific activity 58 mCi mm−1, at a final concen-
tration of 82 μM. Total enzyme activity in the foot was
calculated taking into account the different volumes of feet
different ages. Control experiments ensured that activi-
ties were independent of the volume of homogenization
buffer and that the assay was linear. Specificity of enzyme
activity was tested by running assays in the presence of
4 × 10−3 M 4-(1-naphthylvinyl) pyridine (Calbiochem), said
to be a specific inhibitor of ChAT (White & Cavallito,
1970).

All embryos from at least two litters were examined on
each embryonic day from E15 to E20 inclusive; numbers of
tissues sampled are given in Table 1. Motoneurone death
was inhibited by placing slow-release capsules containing
TTX into the amnion of individual embryos at E15 (Harris,
1981; Harris & McCaig, 1984). The criterion for successful
paralysis was complete flaccidity of the embryo and a lack
of reflex response to electrical stimulation of the snout.

Results

Development of the IVth lumbrical muscle in the rat
hindfoot is similar to that described by Kelly & Zacks
(1969a) for intercostal muscles, except that each
developmental stage is retarded by 2–3 days. The first
signs of lumbrical muscle formation were seen on E15
when a condensation of mesenchymal cells appeared
between two whorls of connective tissue running up
into the IVth and Vth digits. Axons appeared, singly
and in bundles, within this condensation and fre-
cently made close contacts (<20 nm) with the undif-
erentiated cells. At these points of contact, some
axons showed a local enlargement lying in a surface
depression on the mesenchymal cell while others had
no such specializations.

One day later (E16), a few small (3–5 μm) diameter
myofilament-containing cells were present within the
muscle mass. These were the earliest primary myo-
tubes. They were irregular in cross section with
central nuclei, modest numbers of myofilaments dis-
persed throughout the cell, and small deposits of lipid
and glycogen. Small patches of amorphous material,
preumably a rudimentary basal lamina, occurred on
the plasmalemma. Large numbers of axons were seen
within the muscle and these made prolific contacts
with both myotubes and undifferentiated cells. Dur-
ing the period E16–E18, intramuscular axons could
be defined as belonging to motor or sensory neurones
only rarely, when motor nerve terminals contained
synaptic vesicles.

By E17, the primary myotubes had enlarged and
contained abundant myofilaments, lipid and glycogen.
They occurred in groups of three to eight cells
with an incomplete basal lamina irregularly surround-
ing the group. Gap junctions between myotubes were
frequent. Large numbers of axons coursed through
the muscle, while nerve–myotube junctions were still
simple appositions between an unspecialized region
of sarcolemma and a varicosc nerve terminal contain-
ing an occasional dense-core vesicle. Two primary
myotubes sometimes shared an individual nerve ter-
ninal. Fibroblasts, distinguished as elongated cells
containing abundant rough endoplasmic reticulum,
were also present within the muscle, along with
irregularly shaped undifferentiated cells. The latter
had a moderately dense cytoplasm and heterochro-
matic nuclei and are presumed to be a mixture of
myoblasts and fibroblast precursors (Quinn, Namero-
off & Holtzer, 1984).

During the next day (E18), the primary myotubes
(Fig. 1A) progressively separated from their clusters,
but no new cells appeared. On E19, however, small
myofilament-containing cells were seen beneath the
basal lamina of a few of these separate primary
myotubes (Fig. 1B). These were the earliest second-
ary myotubes and their number progressively
increased during the remainder of the embryonic
period. At E19, motor nerve terminals could be
identified lying outside the basal lamina of the muscle
cells while sensory nerve terminals lay close to the
plasmalemma. Muscle spindles were first obvious on
E20. They were distinguished by the capsule sur-
rounding the midregion of the intrafusal fibre and by
the presence of sensory nerve terminals; the IVth
lumbrical muscle typically contained two or three
spindles. By E21, most primary myotubes had one
to three associated secondary myotubes of varying
maturity (Fig. 2A). Undifferentiated cells, presum-
ably myoblasts, were present beneath the basal lam-
ina of primary myotubes. In addition, medium-sized
myotubes began to appear in isolation (Fig. 2A): these
cells are thought to be secondary myotubes
which have separated from their primary clusters
(Ontell & Koze, 1984a). By E22, it became difficult
to distinguish between primary and maturing secondary
myotubes, except on the basis of the presence or
absence of associated young myotubes.

The maturation of nerve–muscle junctions oc-
curred in a way similar to that described by Terävä-
nen (1968) and Kelly & Zacks (1969b). We noted the
presence of close contacts between nerve terminals
and mononucleate cells (Fig. 2B) during the period
when secondary myotubes were forming.

Time course of myotube generation

All myotubes in the endplate region of lumbrical
muscles were counted in a series of muscles aged from
Fig. 1. Initiation of secondary myotube formation. (A) Cross section of a cluster of four primary myotubes in an E18 lumbrical muscle, one day before secondary myotubes are first seen. (B) Cross section of an E19 lumbrical muscle. Primary myotubes have separated and a young secondary myotube (2°) is present adjacent to one of the primaries. Bars, 1 μm.
Fig. 2. Secondary myotubes and muscle innervation; cross sections from E21 lumbrical muscles. (A) A cluster of muscle cells, including a primary myotube with two secondary myotubes (2°) and a mononucleate cell (mn) (presumably a myoblast) closely adherent to it inside the basal lamina. At right, a third secondary myotube has separated from the parent cluster. (B) Contact between a nerve terminal (arrow) and a mononucleate cell. At left, a group of axons makes multiple synaptic contacts with a primary myotube (1°). Bars, 1 μm.
E15–E22 inclusive (Fig. 3) (see Table 1 for the number of muscles sampled). Primary myotube generation began after E15 and reached a plateau of 102 ± 4 cells by E17. No further myotubes were generated for two days, while the existing myotubes matured. A few secondary myotubes (4 ± 0.8, n = 9) were seen for the first time on E19, presaging a period of rapid generation; 65 secondaries, on average, were added between E19 and E20; 100 between E20 and E21; and 114 between E21 and E22. Myotube generation was far from complete by birth: six muscles from PN28 rats contained 919 ± 15 muscle fibres; hence about 530 secondary myotubes were added postnatally. Betz et al. (1979), in a different strain of rat, counted an adult number of 938 ± 79 lumbrical muscle cells.

**Undifferentiated mononucleate cells**

The number of myoblasts or presumptive myoblasts in lumbrical muscles of different ages was estimated by counting all undifferentiated mononuclear cells in one midbelly cross section from each muscle (Fig. 4). There was a constant number (mean value 22 ± 2.3) during E16–E18, which then increased during E18–E21 to reach a mean value of 55 ± 4 on E21. The number of undifferentiated mononucleate cells present on each day during E19–21 was well correlated ($r^2 = 0.97$) with the rate of production of secondary myotubes on that day (Fig. 5).

**Time course of L4 axon loss**

The time course of motoneurone death in the lumbar spinal cord was estimated by counting L4 ventral root

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**Fig. 3.** Time course of development of primary and secondary myotubes in the IVth lumbrical muscle. Primary and secondary myotube numbers counted at daily intervals (mean numbers ± s.e.). Secondary myotubes were first seen on E19. O, primary myotubes; □, secondary myotubes.

**Fig. 4.** Number of mononucleate cells in randomly chosen cross sections from the midbelly region of individual IVth lumbrical muscles, counted at daily intervals until E22. Mean numbers ± s.e. Two regression lines are fitted, between E16 and E18, and between E18 and E21, respectively. Cell numbers stay constant between E16 and E18, then increase rapidly as secondary myotube formation is initiated, reaching their peak on E21.

**Fig. 5.** Number of mononucleate cells versus rate of formation of secondary myotubes. The number (mean ± s.e.) of mononucleate cells present each day during E19–E22 is correlated with the mean number of secondary myotubes formed during the preceding 24 h. Correlation, $r^2 = 0.9$. 
axon numbers on successive embryonic days (Fig. 6). The major period of ventral root axon loss was between E16 and E17 when axon numbers fell from 12,000 to 4000 (67% of the total loss). Occasional signs of axon degeneration, in the form of myelin figures and small regions of cellular debris, were seen in the ventral roots over this same period. Axon numbers continued to slowly decline over the next two days before stabilizing at 2175 by E19; overall 81% of axons present on E15 were lost from the ventral root by E21.

**Axon loss at the level of the ankle**

The lumbrical muscles develop later than most proximal muscles in the hindlimb, and their motoneurones make a very small contribution to axons in the L4 ventral root. To obtain a parameter better related to lumbrical motoneurone numbers, we counted axons in all branches of the plantar nerve at the level of the ankle, using one embryo at each day during E16–E19 (Table 2). Growth cones were present in the nerve branches at all these times, indicating that axons were still growing into the foot throughout this period. There was a peak number of axons on E18, which declined by 23% by E19. In one embryo (E17), axons were counted above and below the ankle; there were 8% more axons below the ankle, indicating that some axons had branched. Judging by the large number of axons in the plantar nerve branches, it is likely that the majority was sensory.

**Motoneurone death in the lumbrical motor pool**

Hindfoot ChAT activity progressively increased with increasing embryonic age, except for a marked inflexion during E17–E19 (Fig. 7). This inflexion was not present in the curve of activity versus time for hindfeet from embryos paralysed with TTX, which is known to inhibit motoneurone death (Harris & McCaig, 1984). Accordingly, we interpret this inflexion as reflecting death of a substantial proportion of motor axons projecting to the intrinsic muscles of the hindfoot on E17, with consequent loss of their ChAT activity.
content, while those remaining continued to accumulate ChAT at the normal rate.

**Intercellular junctions: electrical coupling**

A serial and semiserial EM study was made of a single E21 lumbrical muscle to examine the frequency and distribution of intercellular junctions between developing muscle fibres. The muscle was sectioned from the midbelly to the tendon, as described in the methods.

Gap junctions between myotubes were common throughout the entire length of the E21 lumbrical muscle. These junctions consisted of parallel electron-opaque membranes separated by a gap of 2–4 nm. The entire junctional complex was about 15 nm wide, similar to that reported by Schmalbruch (1982) in newborn rat lumbrical muscles. The distribution of gap junctions between cells varied according to the maturity of the myotubes. Young secondary myotubes within clusters were always extensively electrically coupled to the parent primary myotube along their entire length. More mature secondary myotubes, which had separated from the primary myotubes in their midzone, were still extensively coupled to the primary by gap junctions located near the ends of the secondary myotubes. Even the most mature secondary myotubes present in the E21 muscle still rejoined their primary cluster for some part of their length, and gap junctions between primary and secondary myotubes were commonly seen in these regions.

Gap junctions between neighbouring primary myotubes were also occasionally observed, close to the tendon. In two cases, junctions were seen between mononucleated cells and a filamented cell, and on a single occasion, between two mononucleated cells. Gap junctions involving mononucleated cells were less electron dense than those between myotubes and their apparent rarity may result from difficulties in identifying them.

**Intercellular junctions: mechanical coupling**

Mechanical attachment of developing myotubes to tendons was also examined in the serial section study. At E21, primary myotubes had a classical insertion onto the collagen fibrillar network which needs no further description here. Secondary myotubes, however, rarely inserted directly onto the tendon but at most stages of maturity were attached only to the primary myotube of their cluster. Along much of the length of the secondary myotube, numerous finger-like processes from the cell delved deeply into the primary myotube, forming an intricate attachment between the cells (Fig. 8). Desmosome-like structures were common between secondary and primary myotubes in these regions. These junctions had parallel electron-dense membranes separated by a regular gap of about 25 nm, but the sub-membranous dense plaques, which are a feature of classical desmosomes, were never apparent.

Only the most mature secondary myotubes, those which had separated from the primary cluster for most of their length, formed direct attachments to the collagenous network of the muscle tendon. Even these myotubes, at this age, maintained some small regions of attachment to the primary myotube.

**Discussion**

This study extends those of Kelly & Zacks (1969a); Ashmore et al. 1972; Ontell & Dunn (1978); Ontell & Kozeka (1984a,b) and others, by tracing and quantifying mammalian myotube production and differentiation from the earliest times, and is the prelude to a study on the mechanism of regulation of muscle fibre numbers.

**Time course of generation of myotubes**

Primary myotube generation in the lumbrical muscle begins after E15 and is complete by E17. No new myotubes are added for a further two days, until the first secondary myotubes appear on E19. A similar delay between primary and secondary myotube generation has been described in the mouse extensor digitorum longus (Ontell & Kozeka, 1984a). Ontell & Kozeka (1984b) suggest that primary myotubes require at least 2 days to mature sufficiently for them to act as scaffolding for secondary myotube formation. A contrasting view, supported by the experiments of White, Bonner, Nelson & Hauschka (1975), Rutz, Haney & Hauschka (1982) and Quinn et al. (1984), is that the myoblasts that fuse to form primary and secondary myotubes descend from different classes of myogenic stem cell, so that the time delay reflects the absence of activated secondary myotube precursor cells.

More than half the lumbrical muscle secondary myotubes are added after birth. Postnatal formation of myotubes has been suggested to be common in rat development (Chiakulas & Pauli, 1965; Rayne & Crawford, 1975), but electron microscopy (Ontell & Dunn, 1978; Ontell, 1979) shows that in most muscles the mature number of myotubes is present at birth. The muscle fibres are packed in clusters and the apparent proliferation seen with light microscopy is the result of separation of myotubes from these clusters. The hindfoot lumbrical muscles are an exception to this rule and may be among the last muscles in the body to develop.
Accuracy of myotube counts

Primary myotubes run from tendon to tendon from the earliest times of their development (Dennis et al. 1981; Ontell & Kozeka, 1984a). It is therefore valid to assume that all primary myotubes will be seen in a single section across the midbelly endplate-containing region of a developing muscle, regardless of its age. In the lumbrical muscle, these will typically include three myotubes destined to be part of the muscle spindles (these were not counted in the older muscles where they could be identified). In rat extensor digitorum longus muscles, newly formed secondary myotubes were short and at first randomly distributed over the middle two thirds of the muscle so that only 60–70% spanned its point of widest girth (Ontell & Kozeka, 1984a). Lumbrical muscles are fusiform so that muscle fibre–tendon relations are simpler than in the pennate EDL and, in the serial section study of an E21 lumbrical muscle, all secondary myotubes were centred on the endplate zone. Thus, our counts would include all secondary myotubes more than a few hours old.

Muscle cell death has a role in muscle morphogenesis, but we did not see degenerating myotubes in lumbrical muscles during embryogenesis. By analogy with other muscles, it would not be expected until most secondary myotubes had formed; our preliminary observations indicate that it occurs after PN10 in the lumbricals and so does not interfere with the accuracy of our counts.

Mononucleate cells

The number of undifferentiated mononucleate cells in a single muscle cross section was used as an estimate of the number of myotube precursor cells in the muscle. The cells counted include myoblasts and myoblast and fibroblast precursor cells; at present, it is not possible to distinguish between these cell types.

Fig. 8. Attachment of a secondary myotube to the primary myotube. In this cross section of an E21 lumbrical muscle, a secondary myotube (2°) lies within the basal lamina of a primary myotube (1°). Long processes of the secondary myotube push deeply into the primary myotube. At the interface between the myotubes is a junction displaying parallel, electron-dense membranes separated by a regular cleft (arrow); such junctions are thought to be a form of adhering junction ('desmosome-like' junction). mn, mononucleate cell. Bar, 1 μm.
The increase in number of these cells during the last four days in utero, concomitant with the appearance of secondary myotubes, we take to reflect an increased proliferation of myoblasts.

Myogenic cells have at least three possible fates: to give rise to primary myotubes, secondary myotubes or muscle satellite cells (Armand, Boutinou, Mauger, Pautou & Kieny, 1983). Whether these classes constitute a single lineage or reflect the presence of two or several stem cell lines is not known (Quinn et al. 1984), although it is clear that all are of somitic mesodermal origin (Armand et al. 1983).

**Junctions between myotubes**

Gap junctions between primary myotubes were prominent from their time of first appearance on E16. They have been observed from the earliest times of myotube development in rat intercostal muscles (Kelly & Zacks, 1969a; Dennis et al. 1981), rat hindlimb muscles (Rash & Stachelin, 1974; Schmalbruch, 1982) and in amphibian myotomes (Blackshaw & Warner, 1976), as well as in myoblast and muscle cell tissue cultures (Rash & Fambrough, 1973). Coupling is so extensive that excitation spreading laterally between myotubes gives rise to waves of excitation that propagate across the entire muscle (Dennis et al. 1981). Thus, all the coupled myotubes are subject to the same pattern of activity. Gap junctions may also be involved in the transfer of regulatory molecules between cells (Gilula, Reeves & Steinbach, 1972).

Secondary myotubes in the lumbar muscle were all joined to primary myotubes via gap junctions at E21, and so can be assumed to be electrically coupled. Also they were mechanically coupled to the primary myotubes, via interdigitating insertions which pushed deeply into the primary myotube and by desmosome-like junctions. The structure of these desmosome-like attachment zones between primary and secondary myotubes closely resembled that of the fascia adherens seen in the intercalated discs of cardiac muscle.

**Muscle innervation**

In amphibia, birds and mammals, axons enter the premuscle mass before myotube formation begins (Filogamo & Gabella, 1967; Teräväinen, 1968; Bennett & Pettigrew, 1974; Kelly & Zacks, 1969b; Dennis et al. 1981; Cameron & McCredie, 1982). These observations are confirmed here. The failure of Ontell & Kozeka (1984a) to see axons in the EDL premuscle mass might reflect difficulties in fixation.

Nerve–myotube contacts were observed from the outset of appearance of myotubes. Dennis et al. (1981) observed that nerve stimulation evoked muscle contraction from the earliest time of appearance of primary myotubes and so we assume that the axon terminals seen on E16 in rat lumbral were functional; at this time, they were growth cones containing occasional vesicles and the myotube membrane was un specialized at the point of apposition. A study of the initiation of nerve contacts on secondary myotubes is presented separately (Duxson, Ross & Harris, 1986). Maturation of nerve–muscle junctions in lumbral muscles was similar to that in rat intercostal muscle (Teräväinen, 1968; Kelly & Zacks, 1969b), apart from the difference in timing, and needs no further description here.

**Nerve contacts with mononucleate cells**

Nerve–undifferentiated cell contacts were abundant in E16 lumbricals; these may have been maintained during fusion of myoblasts to form myotubes. Close contacts between nerve terminals and nonfilamented cells also were present during the period of secondary myotube formation, E19–22 (Fig. 2B). Serial section analysis confirmed that these cells were mononucleate and contained no myofilaments (M. Duxson, unpublished observations). Contacts between axons and undifferentiated cells have been seen in other tissues and species including chick myotomes (Sisto Daneo & Filogamo, 1973); chick ALD and PLD muscles (Oppenheim & Chu-Wang, 1983) and rat intercostal muscle (Kelly & Zacks, 1969b). The destinies of these undifferentiated cells (muscle or connective tissue) and the identity of the axons (motor or sensory) are generally unknown.

**Motoneurone death**

Motoneurone death is described in the chick embryo by Hamburger (1948), and has recently been quantified both in mice (Lance-Jones, 1982) and rats (Harris & McCaig, 1984). If, as suggested in the following paper (Ross et al. 1987), innervation is involved in regulating the number of myotubes generated in a muscle, it is important to understand how motoneurone death is regulated so as to determine how many axons will supply the muscle during the period of generation of secondary myotubes.

Death of lumbral motoneurones was monitored by assaying total ChAT activity in the hindfoot over time. Cairns, McCaig & Harris (1986) found a linear relation between the number of axons in the phrenic nerve and E21 rat diaphragm muscle ChAT activity, and we assume the same relation between muscle nerve axon number and muscle ChAT activity to hold during development of the hindfoot muscles. An inflexion in the ChAT activity curve indicates loss of a significant proportion of the axons innervating the hindfoot muscles over E17–E19. Motoneurone death in the lumbar spinal cord was assayed by counting L4
ventral root axons. The L3, L4 and L5 adult ventral roots have similar numbers of axons (Coggeshall, Emery, Haruhide & Maryward 1977) thus minimizing any risks of inaccuracy due to misidentification. Most motoneurone death in the lumbar spinal cord occurred during E16–E17 and was substantially complete by E19.

The lack of synchrony between removal of nerve terminals in muscles of the foot and the general timecourse of motoneurone death in the lumbar cord is evidence that death in an individual motor pool is regulated by feedback from the target muscle. The lumbar is a late-developing muscle, and developmental death of lumbar motoneurones is correspondingly later than most other lumbar motoneurones. At the time of motoneurone death, the lumbar muscles had developed all their primary myotubes, but secondary myotube formation had not yet begun. All motoneurones that subsequently would innervate secondary myotubes must have survived the cell death period by contacting primary myotubes. If survival of motoneurones is determined by competition (Easter, Purves, Rakic & Spitzer, 1985) within a 'target', this target consists solely of primary myotubes, representing a small fraction of the number of adult muscle fibres (10% in the case of lumbricals) or of adult synaptic sites. The role of motoneurone death, we suggest, is not to adapt the number of motoneurones to the number of synaptic sites, but to regulate the number of motoneurones in order to determine the ratio of secondary to primary myotubes in individual muscles.

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