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THE ORGANIZATION AND DEVELOPMENT OF COMPARTMENTALIZED INNERVATION IN RAT EXTENSOR DIGITORUM LONGUS MUSCLE

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SUMMARY

1. We have examined the innervation of the rat extensor digitorum longus (EDL) muscle by the two extramuscular branches formed from the bifurcation of its muscle nerve. Observations of muscle contractions, recordings of end-plate potentials, and glycogen depletion of young adult muscles show that each branch innervates a separate region or 'compartment' in the muscle. The branch entering the muscle nearer the knee (the K branch) innervates fibres in the anteromedial half of the muscle whereas the branch entering closer to the foot (the F branch) innervates fibres located posterolaterally. Individual EDL motoneurones project either into the K or the F branch and therefore innervate fibres located in one compartment. The boundary between the compartments is usually sharply delineated. No obvious anatomical feature exists within the muscle which would explain the division of the muscle into two distinct regions.

2. The presence of a segmentotopic projection from the spinal cord to the muscle was investigated to evaluate its possible contribution to the compartmental pattern. The most posterior neurones of the EDL motor pool were found to project more frequently to the posterolateral F compartment; similarly, the most anterior neurones most frequently project to the anteromedial K compartment. However, each compartment is innervated by both anteriorly and posteriorly located motoneurones. The segmentotopic projection is too weak to explain the presence of neuromuscular compartments.

3. The post-natal period of synapse elimination appears to play at best a minor role in setting up the compartmentalized innervation. Glycogen depletion and intracellular recording in 1–2-day-old muscles show that each nerve branch innervates fibres in the same region of the muscle as in the adult. Most of the fibres in each compartment are polyneuronally innervated by axons in their own particular nerve branch, although fibres located near the boundary between the two compartments are innervated by axons from both nerve branches. This convergent innervation from the two branches disappears in concert with the elimination of

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polyneuronal innervation throughout the muscle. A random elimination of these convergent inputs appears adequate to explain the final compartmental pattern.

4. Our findings suggest that the compartmental pattern is primarily the consequence of the segregation of EDL motoneurones into two nerve branches which are directed into separate regions of the muscle.

INTRODUCTION

The motor unit is generally considered to be the subdivision of skeletal muscle that the nervous system uses to effect a particular motor output. A number of individual motor units are recruited to generate a particular amount of force during a given motor task. There is a growing body of evidence, however, that indicates that the nervous system recognizes further subdivisions in skeletal muscles which are larger than the motor unit. These have been termed 'compartments' (Letbetter, 1974; Botterman, Binder & Stuart, 1978). Anatomically, a neuromuscular compartment has been defined as a contiguous group of muscle fibres and muscle receptors that are innervated by a branch of a muscle nerve (English & Letbetter, 1982*a*, *b*). Functionally, evidence from electromyographic (EMG) recordings suggests that, in some instances, compartments may be activated independently (Herring, Grimm & Grimm, 1979; English & Letbetter, 1981; Russell, Dunbar, Rusher, Macpherson & Phillips, 1982; English, 1984). In addition, muscle compartments may be related to a localization of the stretch reflex (Binder, 1986). Muscle spindles and tendon organs in some muscles have been shown to be more responsive to the activation of motor units in the same compartment than other compartments in the same muscle (Botterman *et al.* 1978; Cameron, Binder, Botterman, Reinking & Stuart, 1981; Hamm, Botterman, Reinking & Stuart, 1981; Lucas & Binder, 1984). Furthermore, Ia afferent fibres innervating muscle spindles within a compartment tend to provide somewhat stronger monosynaptic input to motoneurones innervating muscle fibres in the same compartment (Botterman, Hamm, Reinking & Stuart, 1983; Lucas & Binder, 1984).

Despite an understanding of some aspects of compartmentalized muscle innervation in the adult, how the compartmental pattern arises during development is unknown. Embryonic and neonatal muscle fibres are unlike their adult counterparts in many respects, not the least of which is that they are innervated at a single synaptic site not by one but by several motoneurones (Redfern, 1970; Brown, Jansen & Van Essen, 1976). This polyneuronal innervation is gradually reduced during the first weeks of post-natal life until most muscle fibres become singly innervated. Whether this process, commonly referred to as synapse elimination, plays any role in establishing qualitatively correct synaptic connections in muscle remains unclear. In the course of an examination of synapse elimination in the fast-twitch extensor digitorum longus (EDL) muscle of the rat, we noted several anatomical and histochemical features which suggested that this muscle might be compartmentalized. We have used glycogen depletion, intracellular recording of end-plate potentials and recordings of muscle contractions to identify neuromuscular compartments in young adult EDL muscles and have further investigated the role

of post-natal synapse elimination in the generation of the compartmental pattern of innervation.

A preliminary report of some of these results has appeared in abstract form (Balice-Gordon & Thompson, 1985).

METHODS

The Wistar rats used in this study, the methods for muscle and nerve dissection, for indirect and direct muscle stimulation, for *in vitro* measurements of muscle tension and for intracellular recordings of end-plate potentials are described fully in the preceding paper (Balice-Gordon & Thompson, 1988). Only methods specific to the present study are included here.

Glycogen-depletion experiments. One of the two extramuscular nerve branches to EDL was severed near the point of bifurcation. Each nerve branch was drawn into a suction electrode for stimulation. Isometric twitch or tetanic contractions produced by simultaneous stimulation of the branches were compared with those produced by stimulating the muscle directly. The overlap of territory between the branches was calculated as the difference between the sum of the tension elicited by separate stimulation of the nerve branches and the tension elicited by direct stimulation of the muscle. Tension measurements are expressed as the average percentage of direct muscle tension ± standard error of the mean.

The fibres innervated by the nerve branch remaining connected to the common peroneal were depleted of their glycogen using a modification of the procedure of Kugelberg (1973). The stimulation frequency at which maximal tetanic tension was obtained was determined. For an EDL from a 15-day-old rat this frequency was typically 50–100 Hz; in newborn animals, it was usually 40–50 Hz. The remaining intact nerve branch was stimulated at this frequency with trains of 500 ms duration; each train was repeated once every 20 s. The tetanic tension tended to decline somewhat (usually less than 10%) as the muscle was stimulated in this fashion; over a period of less than 2–3 min, the tetanic tension reached a stable value. When a steady level of tetanic tension was reached, the superfusion solution was changed to Ringer solution that was bubbled continuously with 95% N_2/5% CO_2 and the train repetition rate was increased to once per second. After the tetanic tension had fatigued to zero (for a 14-day-old EDL muscle, this typically took 5 min), the nitrogenated Ringer solution was immediately replaced with oxygenated Ringer solution and the stimulation regimen returned to one train every 20 s. Each solution and stimulation change typically took less than 10 s. The tension was allowed to recover to pre-fatigue levels. These fatigue–recovery cycles were repeated 7 to 10 times. After the last cycle, the tetanic tension was not allowed to recover; the muscle was quickly removed from the bath, pinned at resting length between two adult muscles onto balsa-wood sticks, and was frozen in isopentane cooled over liquid nitrogen to −65 to −85 °C.

Histological methods. For histological examination of muscle fibre glycogen content, myofibrillar ATPase, or immunohistochemistry, 10 μm frozen sections were made at −20 °C using a cryostat microtome. For most experiments, sections were made through the mid-belly of the muscle at an angle to its long axis so that the resulting sections included all of the fibres but neither of the muscle's tendons. In another series of experiments, cross-sections were taken at 0·5 mm intervals along the muscle's length to establish the tendons of origin and insertion of the muscle fibres innervated by each extramuscular nerve branch. Alternate sections were then reacted for the presence of glycogen using the periodic acid–Schiff reagent method (after Pearse, 1968) and in some experiments for myofibrillar ATPase following alkaline (pH = 10·4) pre-incubation (Guth & Samaha, 1970). In some experiments, a monoclonal antibody, 1-D-2, which was generated in this laboratory and which recognizes the slow class of myosin heavy-chain isoforms (our unpublished results) was used in immunohistochemistry to mark the type I (SO) fibres in this muscle. Another monoclonal antibody also generated in this laboratory, 10-C-8, which stains connective tissue and tendon (our unpublished results), was used in some experiments to check for the presence of intramuscular tendinous inscriptions. Anti-mouse secondary antibodies, either fluorescein conjugated, or horseradish peroxidase conjugated followed by a diaminobenzidine reaction, were used to visualize the pattern of staining. Muscle fibre counts were made from photomontages of entire muscle cross-sections in some experiments and are expressed as an average ± standard error

of the mean. Serial reconstructions were made from camera lucida drawings. A silver stain according to the procedure of Winkelmann and Schmit (Kiernan, 1981) was used on whole mounts of 1 day and 14 day EDL muscles to demonstrate the intramuscular pattern of nerve branching. The method of Buckley & Heaton (1968) for visualization of acetylcholinesterase was used to determine the location of neuromuscular end-plates.

RESULTS

Compartmentalized innervation in young adult EDL muscles

The extensor digitorum longus (EDL) is a pinnate, fusiform muscle often utilized in experimentation as a representative fast-twitch muscle. The anatomy of the muscle and its nerves in the rat is illustrated in Fig. 1. EDL muscle fibres originate from one tendon attached to the lateral epicondyle of the distal femur. Towards the ankle, the muscle fibres divide into four groups, each of which attaches to a separate tendon. After passing through the annular and calcaneal ligaments, each of the four tendons inserts on the distal phalanx of one of four digits of the foot (Fig. 1*A*). The fascicular outlines of the four groups of muscle fibres can be followed from tendon to tendon when the muscle is pinned in a preparation dish with the four tendons dissected apart (Fig. 1*B* and *C*). The four fibre groups arise from the tendon at the knee in a roughly proximal-to-distal order, with the largest group of fibres, those inserting on the tendon to digit 5, arising the most distally. In the rat, the EDL muscle nerve, a branch of the common peroneal, bifurcates into two primary extramuscular nerve branches just outside the muscle. One of these branches enters the muscle on its posteromedial surface about one-third the length of the muscle from the proximal tendon (Fig. 1*C*). The second branch enters the muscle more distally on its posterior surface. The first branch, entering the muscle closer to the knee, is designated here the K branch and the second, entering the muscle closer to the foot, the F branch. The two primary branches were usually separated in their point of entry in the muscle by approximately one-third the muscle fibres' length and one-quarter of the muscle's circumference. In most EDL muscles, these primary branches bifurcated again as they entered the muscle; these smaller, secondary branches were usually inaccessible for further experimentation.

In the course of an investigation of synapse elimination in rat EDL, we became interested in whether the two primary extramuscular nerve branches might, as has been observed for other muscles with branched nerves, innervate discrete muscle regions or 'compartments'. In an initial set of experiments, the contribution of each nerve branch to muscle innervation was assessed by stimulating each branch and observing through a dissecting microscope contractions of the muscle pinned to the bottom of a preparation dish. These observations suggested that EDL is indeed compartmentalized: stimulation of the K branch evoked contractions in the anteromedial region of the muscle, whereas stimulation of the F branch evoked contractions in the muscle's posterolateral aspect. Measurements of muscle tension showed that each branch innervated roughly half of the muscle, although the contributions of each branch varied somewhat from muscle to muscle. In twelve 14–19-day-old muscles, the K branch tension was $52 \pm 3\%$ of the direct twitch tension whereas the F branch tension was $57 \pm 3\%$. In some muscles, stimulation of

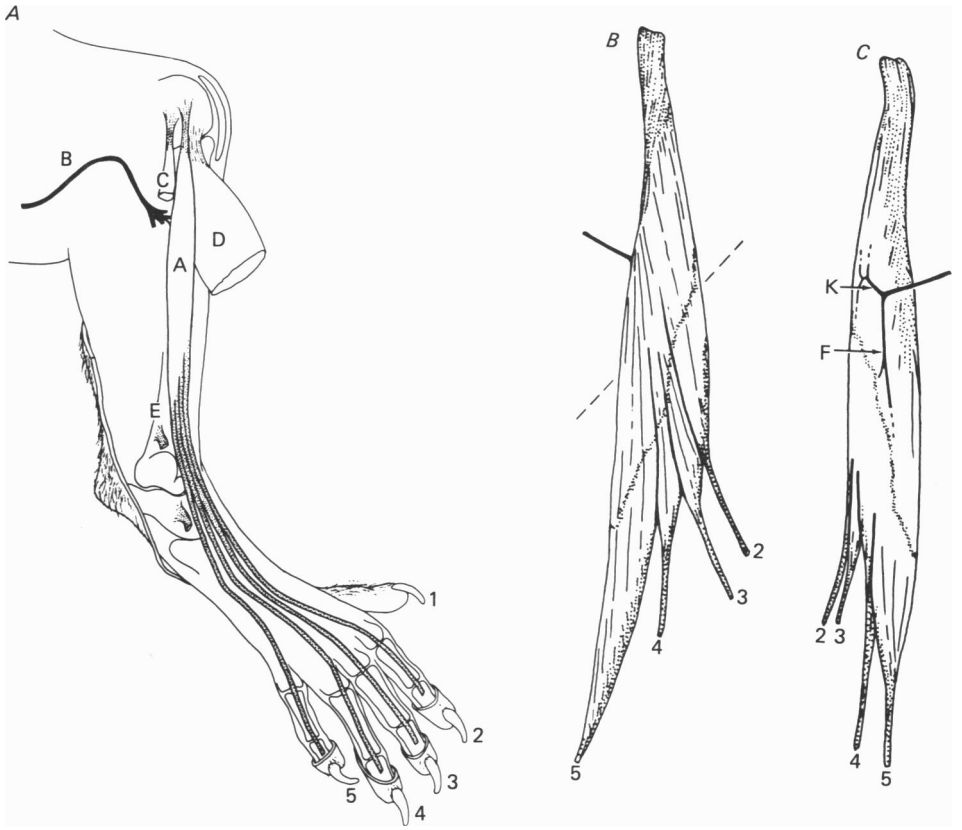

Fig. 1. Anatomy of the extensor digitorum longus muscle (EDL) of the rat. *A*, location of EDL in the lateral portion of the hindlimb. EDL (A) is located lateral to the tibia (E), anterior to the peroneus longus muscle (C) and is immediately posterior to the tibialis anterior muscle (D). The muscle's tendon of origin (stippling) is attached to the lateral epicondyle of the distal femur. The four tendons of insertion pass under the annular and calcaneal ligaments (shown severed; unlabelled), travel over the surface of the foot and attach to the distal phalanx of digits 2, 3, 4 and 5. The common peroneal nerve (B) supplies the innervation to this muscle via two primary extramuscular nerve branches (below). *B*, arrangement of EDL muscle fibres, lateral surface of muscle. Orientation as in *A*; the lateral surface of the muscle is shown, the tendon of origin is at the top, anterior is to the right and posterior to the left. The tendons of insertion have been dissected apart. The muscle fibres are organized into four groups. The largest group arises most distally from the tendon of origin and attaches to the tendon of digit 5. The end-plates (shown as dots) were visualized by cholinesterase staining and are located in a single continuous plane half-way between the tendons of origin and insertion. The dashed line indicates the level and angle at which sections were made so as to include all of the fibres in the muscle. *C*, location of primary nerve branches. The muscle in *B* has been rotated around its long axis 90 deg to the right to show the posterior surface. The lateral surface of the muscle is now to the right and the medial surface to the left. The deep branch of the common peroneal nerve divides into two primary extramuscular nerve branches, designated K and F.

the K branch generated as little as 38% or as much as 67% of total muscle twitch tension. A sample of some of these data is shown in Table 1.

To verify the visual impression that the two nerve branches innervated segregated groups of muscle fibres, intracellular recordings of end-plate potentials evoked in response to stimulation of each branch were made from four 13–14-day-old EDL

TABLE 1. Compartment measurements from K branch glycogen-depletion experiments

	Tension measurements (% of direct muscle twitch)		Area of K compartment (% of total muscle area)	Number of depleted and undepleted fibres in region of intermixture
	K branch	F branch		
14-day-old muscles*				
No. 1	51	53	48	860
No. 2	61	46	55	780
No. 3	56	51	52	952
No. 4	52	58	63	1157
No. 5	38	74	42	924
Average	51	56	52	935
S.E.M.	2	2	3	73
1–2-day-old muscles†				
No. 6	59	64	52	520
No. 7	42	65	45	558
No. 8	69	50	65	900
No. 9	50	76	50	936
No. 10	79	57	70	603
No. 11	65	39	55	392
No. 12	64	63	59	568
Average	61	59	56	640
S.E.M.	5	5	3	76

* Data are shown for five of the seven EDL muscles depleted through the K nerve branch at 14 days. The two muscles not included had cross-sections which contained tendon and were therefore judged not to include all of the muscle fibres.

† Data are shown for seven of eight EDL muscles depleted through either nerve branch. The muscle not included was depleted through the F branch.

muscles. At least fifty fibres were sampled across the medial surface of the muscle. In three of these muscles fibres across the lateral surface were also sampled. In confirmation of the visual observations, these intracellular recordings showed that fibres in the posterolateral region of the muscle are innervated by axons in the F branch while fibres in the anteromedial portion of the muscle are innervated by the K branch. In agreement with the variability found in the tension measurements, as many as 80% and as few as 40% of the total sample were found to be innervated by axons in the K branch. At the boundary between these two regions, fibres innervated by one or the other nerve branch were intermixed. Furthermore, some fibres innervated by axons in both branches were found in this region. These fibres represent a portion of the EDL muscle fibres which remain polyneuronally innervated at 14 days (Balice-Gordon & Thompson, 1988).

The technique of glycogen depletion was used in eleven 14-day-old muscles to determine more precisely the arrangement of fibres innervated by either the K (seven experiments) or the F branch (four experiments). In each experiment, one branch was subjected to repeated tetanic stimulation. Following this stimulation procedure, frozen, transverse sections of the muscle were stained for the presence of glycogen. Examination of sections made at an angle through the muscle so as to include all of

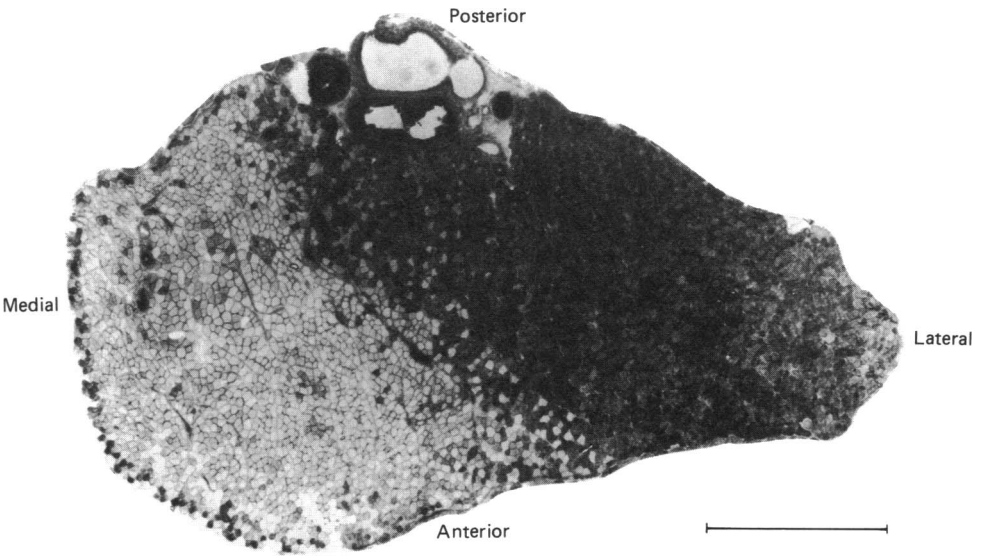

Fig. 2. Location of neuromuscular compartments in a 14-day-old EDL muscle. Muscle cross-section made at an angle to the muscle's long axis (dashed line in Fig. 1*B*) so as to include all muscle fibres but neither of its tendons. Section stained for glycogen with the periodic acid–Schiff reaction. The fibres innervated by the K branch have been depleted of their glycogen with repetitive tetanic stimulation and appear white in the photograph. The fibres innervated by the F branch were not stimulated, remain undepleted, and appear dark in the photograph. Each primary extramuscular nerve branch innervates a distinct subvolume or compartment of the muscle. The undepleted fibres at the periphery of the K compartment are an occasionally observed artifact of the depletion (Iliya & Dum, 1984). The K branch compartment occupies 48% of the muscle's territory. The boundary between the two compartments is distinct, although depleted and undepleted fibres are intermingled across it. There are 860 depleted and undepleted fibres intermingled in the boundary area. Tension and muscle territory measurements for this experiment are listed in Table 1 as experiment No. 1. Bar = 500 μm.

the muscle fibres (six experiments) revealed about half of the fibres in the muscle stained intensely for glycogen and the remaining half stained weakly or not at all (Fig. 2). Moreover, the depleted (and undepleted) fibres occupied contiguous subvolumes, or compartments, of the muscle. Muscles stimulated through the K branch consistently showed depleted fibres in the anteromedial half of the muscle (Fig. 2), whereas those stimulated through the F branch showed depletion in the posterolateral half of the muscle. The location of the compartments was reproducible from experiment to experiment.

An estimate of the size of each compartment was made by counting the number

of depleted and undepleted fibres in photomontages. Because in some muscles it was difficult to obtain an accurate count from 'periodic acid–Schiff' sections, the depleted and undepleted areas were drawn onto tracing paper, and an estimate was made of the proportion of fibres in each compartment by weighing the tracings individually and together. Both methods were applied to some muscles and excellent agreement was obtained (data not shown). Comparison of tension data with the size determinations made after glycogen depletion also showed good agreement. While there appears to be variation from muscle to muscle in the exact size of the compartments, on average, each nerve branch innervates roughly half of the muscle (Table 1).

In order to determine the relationship of each compartment to the groups of fibres inserting on the four distal tendons of the muscle, in five glycogen-depletion experiments sections were made perpendicular to the long axis of the muscle at 0·5 mm intervals. Because of the tendon arrangement in EDL, varying numbers of fibres were seen in these cross-sections, depending on the level of section. Accordingly, a variable proportion of depleted or undepleted fibres was observed: near the proximal tendon most of the fibres present are in the K compartment whereas near the distal tendon most of the fibres present are in the F compartment. Reconstructions of the muscle from these sections indicated that the muscle fibres which inserted onto the tendon to digit 5 are innervated almost exclusively by axons contained in the F branch. However, following stimulation of either the K or F branch, a mixture of depleted and undepleted fibres was found on all of the remaining toe tendons, suggesting that there is no consistent relationship between the compartments and the muscle fibres inserting on digits 2–4.

In each experiment there was a reasonably sharp and distinct boundary between the depleted and undepleted regions which ran from the anterolateral to the posteromedial aspect of the muscle (Fig. 2). In the region of this boundary, depleted and undepleted fibres were intermixed to varying degrees. To obtain a rough estimate of this intermixture of fibres innervated by the K or F branch, a parallelogram which contained the undepleted fibres found in the depleted compartment and the depleted fibres found in the undepleted compartment was drawn on each muscle cross-section. The long sides of this parallelogram were parallel to a line which defined the most distinctive border between the two compartments. The number of fibres along this line and one of the short sides of the parallelogram were counted and the product of the two was taken as a measure of the boundary area. This procedure was applied only to muscles in which all of the muscle fibres were present in a single cross-section; however, the degree of intermixture was not more pronounced in the remaining muscles in which complete cross-sections were not obtained. In the five 14-day-old muscles which met this criterion, this area was on average 933 muscle fibres (Table 1); in the experiment illustrated in Fig. 2, the boundary area was estimated to contain 860 fibres.

Projections of EDL motor axons into the K and F nerve branches

The above experiments show that each branch of the EDL nerve innervates a separate region of the muscle. However, since each experiment involved a preparation in which one of the branches was severed at the point of bifurcation, the

possibility remains that single motoneurones might project into both compartments. To evaluate the projection of the motor axons at the bifurcation, tests were made for axon reflexes. One of the nerve branches was cut near the surface of the muscle and its cut, proximal end, remaining attached to the common peroneal nerve, was drawn into a suction electrode for stimulation. In none of the muscles at 1 day or at 14 days of age did stimulation of the proximal stump evoke detectable muscle tension. Stimulation of the common peroneal nerve, however, evoked about one-half of the tension produced prior to the nerve cut. These results suggest that individual motor axons project into only one of the two branches. Further evidence for this projection pattern was obtained from motoneurone counts. In ten muscles, the total number of EDL motoneurones was determined by counting threshold increments to the twitch tension obtained from graded stimulation of ventral root filaments (Balice-Gordon & Thompson, 1988). The counts were then repeated after one nerve branch was severed. About half of the motoneurones (average twenty-one of forty-one) remained, suggesting that each branch contains an exclusive portion of the muscle innervation. Preliminary experiments in which single motor units have been marked by glycogen depletion (R. J. Balice-Gordon & W. J. Thompson, in preparation) show that motor unit territories are confined to one compartment. Thus, the regionalized innervation in EDL is a compartmentalization of the muscle by single motor units.

Fibre type composition of muscle compartments

Previous investigations have demonstrated that the fibre type composition of muscle regions is often markedly different (Gonyea & Ericson, 1976; Van Winkle, Entman, Bornet & Schwartz, 1978; Galvas & Gonyea, 1980; English & Letbetter, 1982*a*; Bodine, Roy, Meadows, Zernicke, Sacks, Fournier & Edgerton, 1982). The rat EDL is composed of three common mammalian skeletal muscle fibre types: slow-twitch or type I fibres, and two types of fast-twitch fibres, IIa and IIb (Pullen, 1977). A monoclonal antibody generated in our laboratory, 1-D-2, which selectively stains slow fibres, and the histochemical procedure for myofibrillar ATPase were used to examine the fibre type composition and the distribution of these types in rat EDL. In agreement with previous work, we found that an average of $4 \pm 0{\cdot}3$ % (225 of the average 5012 fibres, twelve muscles) of the fibres in 14–101-day-old muscles are slow, type I and that these slow fibres are distributed in an unhomogeneous fashion: slow fibres are concentrated in the medial portion of the muscle and are absent from the lateral portion (Pullen, 1977; Lyons, Haselgrove, Kelly & Rubinstein, 1983). In contrast to the type I fibres, the type IIa and IIb fibres are distributed relatively uniformly throughout the muscle (Pullen, 1977). Because the distribution of slow fibres was clearly not uniform, we determined the slow fibre composition of the two compartments in alternate, serial sections of muscles glycogen depleted through one nerve branch stained either for glycogen to identify the compartments or for demonstration of fibre types. The territory innervated by the K branch, occupying the anteromedial compartment, contains on average 67 % (seven muscles) of the slow fibres present in EDL; the F branch compartment, which occupies the posterolateral portion of the muscle, contains the remainder of the slow fibres. Thus, although there is no marked difference in the fast fibre composition of the two compartments, there is a pronounced disproportion in the slow fibre composition.

Possible mechanisms for segregation of innervation into compartments

There are several straightforward explanations of how the compartmentalization in the rat EDL might arise. Because depleted fibres are found intermingled with undepleted ones all along the boundary, it is clear that axons in one nerve branch are not strictly prevented from innervating muscle fibres in the other compartment. However, a physical barrier at the boundary in the muscle might impede axons crossing between these two regions, resulting in the compartmentalized pattern. Light microscopic examination of 14-day-old muscles in which the boundary region had been defined by glycogen depletion through one nerve branch failed to reveal any tendon or blood vessels which formed such a barrier. In some cases, as illustrated in Fig. 2, a blood vessel or muscle fascicle outline delineated a portion of the boundary, but depleted and undepleted fibres were always found on either side of such a structure. No connective tissue defining these two regions could be detected, even after application of an antibody, 10-C-8, which stains the fascicular connective tissue and tendon (see Methods). Serial reconstructions of 14-day-old muscles confirmed the absence of additional tendinous inscriptions besides the proximal and distal tendons which might define muscle fibre groups corresponding to the compartmental groups. Furthermore, examination of muscle whole mounts which had been stained with silver using the procedure of Winkelmann and Schmit (Kiernan, 1981) revealed that many of the intramuscular nerve branches anastomosed. Taken together, these data strongly suggest that the compartments are not the result of some physical impediment to axons crossing between the two muscle regions.

A second possible explanation for the compartmentalization is a discontinuity of the end-plates at the compartmental boundary. If the end-plates on the fibres in the adjacent compartments were staggered in their location, then synapse formation by axons crossing from one compartment to the other might be unlikely. To examine this possibility, a cholinesterase procedure for demonstration of end-plates (Buckley & Heaton, 1968) was applied to whole, 14-day-old muscles. Examination with a dissecting microscope at a magnification of 50 × showed that the end-plates in EDL are located in a single contiguous band in one plane through the belly of the muscle (Fig. 1); there is no discontinuity in the band in the region of the compartmental boundary or elsewhere in the muscle.

Is the compartmental pattern related to a segmentotopic projection to EDL?

The compartmentalized pattern of innervation in some muscles has been suggested to be related to a segmentotopic projection from the spinal cord to the muscle (Letbetter & English, 1981; Iliya & Dum, 1984; Richmond, MacGillis & Scott, 1985; Weeks & English, 1985). The segmentotopy of the EDL innervation and its relation to the two compartments was examined by determining the projections of anteriorly located (derived from the L4 ventral root) and posteriorly located (derived from L5) motoneurones to the K and F compartments. The number of L4 and L5 motoneurones was first determined by counting motor units in teased ventral root filaments (Balice-Gordon & Thompson, 1988). Either the K or F branch was then cut and the counts repeated. In some experiments the L4 ventral root, which contains

the majority of EDL axons (Balice-Gordon & Thompson, 1988), was split into anterior and posterior halves and the contribution of each to the compartments assessed. In fifteen of twenty-three muscles examined, more of the L5 motor axons projected to the posterolateral, F compartment than to the anteromedial, K compartment (Fig. 3*A*). In eight of nine muscles examined, more L4 axons were

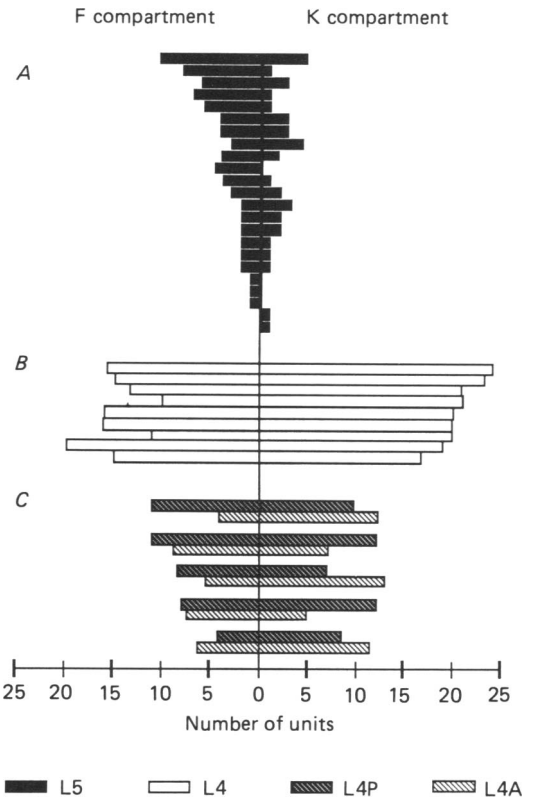

Fig. 3. Segmental origin of motor axons innervating K and F compartments in 14-day-old muscles. The number of L5 axons (*A*) and number of L4 axons (*B*) contained in the K and F branch. *C*, number of motor axons in the anterior (L4A) and posterior halves (L4P) of the L4 ventral root innervating each compartment.

found to project to the K branch compartment than to F. From these observations, there appears to be a weak segmentotopy to the innervation of EDL. However, axons from L4 and L5 innervate both compartments (Fig. 3*A* and *B*). Even when L4 axons are divided into anterior and posterior halves, the projection is mixed to the two compartments (Fig. 3*C*).

Glycogen depletion was employed to examine the segmentotopy of the innervation of the muscle and its compartments more directly. The location of muscle fibres innervated by the most caudal motoneurones in the EDL motor pool was determined by stimulating the motoneurones present in the L5 ventral root in six 14-day-old muscles. Depleted fibres were found to be localized to the posterior portions of the

muscle (Fig. 4). In all of these experiments, however, depleted fibres were intermixed with undepleted fibres, confirming the lack of an absolute segmentotopy. Moreover, although the majority of depleted fibres were located within the F compartment, in each case a portion of the L5 motor axons projected into the anteromedial K compartment. Interestingly, these L5 axons entering the K compartment tended

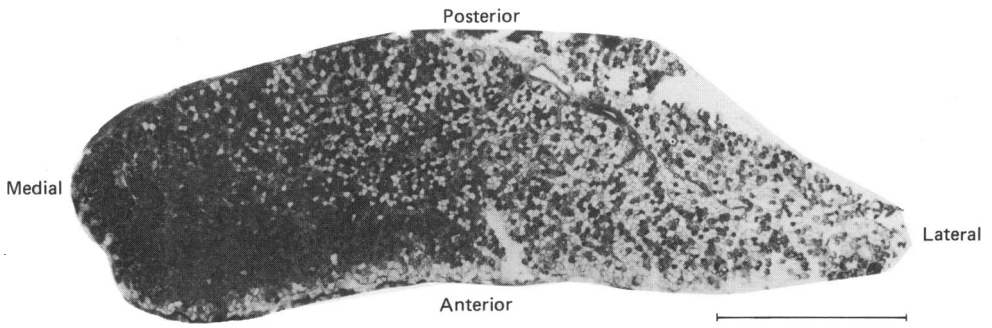

Fig. 4. Projection of L5 axons in a 14-day-old EDL muscle. Muscle fibres innervated by axons contained in the L5 ventral root were visualized after glycogen depletion. There were seven EDL axons in L5; four of these were in the F branch and three in the K branch. Most of the depleted fibres are located in the posterolateral or F compartment of the muscle; however, depleted fibres are located in the posterior portion of the K compartment as well. The small band of depleted fibres at the anterior border of the muscle is an artifact of the application of cyanoacrylate to hold the muscle tendons together (Balice-Gordon & Thompson, 1988). Orientation of muscle section as in Fig. 2. Bar = 500 μm.

to form segmentotopic synapses, innervating fibres in the posterior portions of the K compartment. In any case, these glycogen-depletion experiments together with the experiments determining the compartmental projection of L4 and L5 motoneurones suggest that the segmentotopy alone is insufficient to account for the compartmentalization of the innervation of EDL.

The role of synapse elimination in the organization of muscle compartments

There is an extensive rearrangement of neuromuscular synaptic connections (synapse elimination) which occurs during the first weeks of post-natal life (Redfern, 1970; Brown *et al.* 1976). In rat EDL, all of the muscle fibres are polyneuronally innervated at birth; the first singly innervated fibres appear at post-natal day 3 and it is not until the second week after birth that the majority of the fibres come to be singly innervated (Balice-Gordon & Thompson, 1988). By the end of the second post-natal week, synapse elimination is largely complete; more than 90% of the fibres are singly innervated. To examine the role of the post-natal period of synapse elimination in establishing the compartmental pattern, the innervation of the muscle by each of the primary nerve branches was determined in newborn animals.

Visual observations in newborn muscles showed that, as in older muscles, distinct muscle regions contracted following stimulation of each nerve branch. Muscle tension measurements in eighteen 1–2-day-old muscles also indicated that EDL muscles are

compartmentalized at birth: on average, the K branch generated $67 \pm 4\%$ and the F branch $61 \pm 3\%$ of the direct tetanic tension. Comparable tetanic measurements in 14–19-day-old muscles were 51 ± 3 and $53 \pm 3\%$ (five muscles) respectively, indicating that the muscle territories innervated by each branch are approximately 20% larger at birth than at 14 days. Such enlarged sizes are consistent with the enlarged motor unit sizes present in young EDL muscles (Balice-Gordon & Thompson, 1988) and further suggest that a portion of the fibres in the muscle receive innervation from both nerve branches.

The extent of the convergence of innervation between the two branches was assessed by recording intracellularly from muscle fibres to determine the nerve-branch origin of their synaptic inputs as described above. The results of these experiments in four 1–2-day-old muscles indicated that the majority ($74 \pm 4\%$) of the 180 muscle fibres sampled were polyneuronally innervated exclusively by motoneurones whose axons travelled in one nerve branch or the other. However, $26 \pm 4\%$ of these fibres received synaptic input from axons in both branches. This estimate of convergent innervation from the two branches agrees well with estimates of $28 \pm 5\%$ obtained by determining the tension deficit (the amount the sum of the individual branch tensions exceeded the total tetanic tension elicited by direct stimulation). The initial overlap in innervation between the two branches is reduced as the postnatal period of synapse elimination progresses.

The location of the fibres polyneuronally innervated by each branch corresponded with the position of the compartments in older muscles: muscle fibres polyneuronally innervated by axons in the K branch were usually located in the anteromedial portion of the muscle, while those fibres polyneuronally innervated by F branch axons were located posterolaterally. Furthermore, muscle fibres innervated by both K and F axons were found in the approximate vicinity of the boundary region between the two compartments.

The compartmentalization of newborn EDL muscles was further examined using glycogen depletion in eight experiments on 1–2-day-old muscles. The motor axons in each branch were found to innervate distinct muscle compartments (Fig. 5), which are identical in position to those observed at 14 days. To estimate the proportion of the muscle innervated by the stimulated branch, tracings were made of the depleted compartment and of the entire muscle from photomontages of cross-sections which included all of the muscle fibres. The tracings were weighed and the size of each depleted compartment was expressed as a percentage of the entire muscle (Table 1). Although the sizes varied from preparation to preparation, the depleted compartments were found to be larger at birth than in the adult. For example, measurements of muscle territories in K branch depletion experiments (Table 1) suggested that this compartment was approximately 10% larger at birth than in the adult.

Depleted and undepleted areas of these 1–2-day-old muscles were clearly demarcated in a manner suggestive of the boundary between the compartments in the 2-week-old animals. As in the older animals, there was a small region near the centre of the muscle where depleted and undepleted fibres were intermixed. The extent of this intermixture was estimated as described above for the 2-week-old animals; the intermixture at 1–2 days was generally less than that observed at 2

weeks (Table 1). However, this area measurement does not have the same meaning it does in the 2-week-old animals. At 2 weeks, this measurement estimates the sharpness of the boundary between the compartments. In contrast, the glycogen depletion in the 1–2-day-old muscles gives no information about the boundary between the compartments because this boundary has yet to be established. Rather, fibres near the centre of the muscle receive convergent innervation, and the glycogen depletion shows only those fibres which receive suprathreshold innervation from the stimulated nerve branch. The boundary will emerge somewhere in the depleted area of the muscle as the convergent innervation is eliminated.

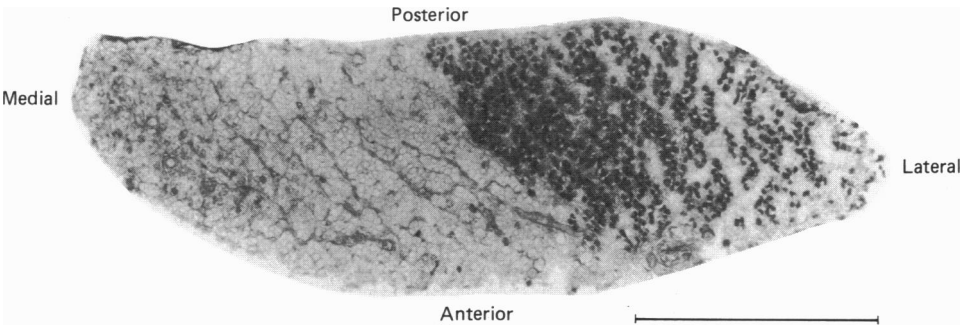

Fig 5. Location of neuromuscular compartments in 1-day-old EDL muscle. Muscle fibres innervated by the K extramuscular nerve branch have been depleted of their glycogen. The compartments are located in the same position in newborn muscle as in 14-day-old muscles (Fig. 2). The K branch territory was 55% of the muscle area. Gaps in the F compartment represent small tears in the section. The region of intermixed depleted and undepleted fibres in this experiment included 392 fibres. Tension and area measurements are included in Table 1 as experiment No. 11. Bar = 500 μm.

These data indicate that the compartmental pattern of innervation is not established by a selective elimination of synapses from an initial random innervation of the muscle. Rather, the basic segregation of innervation into two compartments is present at the time of birth.

DISCUSSION

The term 'compartment' has been used in the literature to describe a variety of regionalized characteristics of muscles. Recently, however, it has been used almost exclusively to describe a group of muscle fibres which occupy a contiguous, reproducible region of a muscle and which are innervated by a branch of a muscle nerve. Using this definition, the compartmental pattern of innervation is relatively common in skeletal musculature (Letbetter & English, 1981; Galvas & Gonyea, 1980; English & Letbetter, 1981; Bodine *et al.* 1982; Iliya & Dum, 1984; Richmond *et al.* 1985). The rat EDL can be added to the list of muscles having this pattern of motor innervation. We have shown here that the motor innervation to EDL is segregated into two distinct regions each innervated by a separate extramuscular branch of the EDL nerve.

Synapse elimination and the compartmental pattern of innervation

One of the major goals of this study was to describe the role of synapse elimination in the generation of compartmentalized innervation in EDL. Because each muscle nerve branch innervates fibres in the same region of the muscle at birth as it innervates in the adult, the basic segregation of innervation into compartments is clearly not the result of an initial random innervation followed by a compartmentally selective synapse elimination. Such a dramatic role for synapse elimination would have perhaps been surprising given previous demonstrations that motor units occupy the same general positions within muscles during this change in innervation (Dennis, Ziskind-Conhaim & Harris, 1981; Brown & Booth, 1983). On the other hand, synapse elimination in EDL does remove the convergent innervation from the two nerve branches present near the centre of the muscle at the time of birth; consequently, synapse elimination reduces the size of each compartment. Still at issue, then, is whether the elimination of this convergent innervation improves the sharpness of the segregation of innervation into compartments over what would be expected from a random elimination.

One could evaluate the possibility of a compartmentally selective elimination of the convergent innervation by the two nerve branches if the location of the fibres receiving this convergent innervation were known. Given this information, it would be possible to make predictions about the consequences of a random synapse elimination for the location of the boundary between the two compartments and for the degree to which fibres innervated by the two nerve branches should be intermixed along this boundary. Unfortunately, glycogen depletion conducted through stimulation of one nerve branch reveals neither the location nor the extent of branch-convergent innervation in the newborn muscles. However, the intracellular recordings and measurements of tension overlap indicate that about 25% of the fibres in the newborn muscle have such convergent innervation. The intracellular recordings further suggest that these fibres occupy a volume of the muscle at the juncture between the two areas innervated exclusively by each nerve branch. It appears likely then that a group of about 1250 fibres (25% of the muscle fibres) is innervated by both nerve branches and is located near the centre of the newborn muscle astride the future boundary. If these 1250 fibres were contiguous, or nearly so, then one would expect that, at the end of synapse elimination, glycogen depletion through either nerve branch would reveal a region of about 1250 intermixed depleted and undepleted fibres located at the boundary between the two compartments. Following glycogen depletion in 14-day muscles a region was observed which contained, on average, 935 intermixed fibres. We do not believe the discrepancy between the number observed and the number expected of a random elimination is a compelling argument for a compartmentally selective synapse elimination. This is especially so since the 1250 fibre value probably overestimates the expected degree of intermixture resulting from a random elimination. Fibres on the K side of the 1250 fibre region would be expected to be innervated by more K than F axons, and, similarly, fibres on the F side of the region would be expected to be innervated by more F than K axons; random synapse elimination would be expected to produce less intermixture at the extremes of this 1250 fibre region. We conclude that there is no

compelling evidence for a selective role of post-natal synapse elimination in the generation of the two neuromuscular compartments in EDL.

This conclusion is in general agreement with findings reported by Iliya & Dum (1984), Donahue & English (1985) and English (1986). Iliya and Dum demonstrated compartments in the tibialis anterior muscle of 6-week-old kittens, near the end of synapse elimination. Although technical difficulties prevented their use of glycogen depletion in younger muscles, they were able to examine the spinal cord location of the motoneurones projecting into each of the two extramuscular nerve branches compartmentalizing the adult muscle. They showed that the motoneurones entering each branch occupied the same relative spinal locations in the neonate as in the adult. Therefore, the projection of motoneurones into the two nerve branches was not rearranged as the result of synapse elimination. Other investigators have used EMG recordings to demonstrate the presence of compartments in neonatal rat lateral gastrocnemius (Donahue & English, 1985) and gluteus maximus muscle (English, 1986). Therefore, the developmental mechanisms which establish compartmentalized innervation appear to be independent of the process which establishes the single innervation of skeletal muscle.

Compartmental innervation in EDL and in other muscles

Several features in addition to innervation by separate nerve branches appear to distinguish neuromuscular compartments. One such feature is a segmentotopically organized projection from motoneurones to compartments: the more anteriorly (or posteriorly) located motoneurones in a compartmentalized muscle's motor pool tend to project to the more anterior (or posterior) compartment (Swett, Eldred & Buchwald, 1970; Letbetter & English, 1981; Iliya & Dum, 1984; Richmond *et al.* 1985; Weeks & English, 1985; Donselaar, Kernell, Eerbeek & Verhey, 1985). This tendency is also apparent in EDL (Figs 3 and 4). However, the segmentotopic projection in EDL is not rigorously organized at the point of bifurcation of the K and F branches, and, as in other muscles, the segmentotopic projection alone cannot account for the strong segregation of motor innervation into compartments.

Compartments in many muscles are defined not only by the pattern of nerve branching but also by the arrangement of tendons in the muscle and/or the presence of intramuscular tendinous inscriptions (Galvas & Gonyea, 1980; English & Letbetter, 1981; Bodine *et al.* 1982; Richmond *et al.* 1985). These tendinous structures may influence the segregation of innervation between compartments. In regions of the cat splenius muscle, for example, compartments are strictly segregated by tendinous inscriptions. However, in other areas of the muscle, where such inscriptions are absent, there is extensive interdigitation of fibres innervated by two nerve branches (Richmond *et al.* 1985). The compartmentalization in EDL is like that in these latter areas of the cat splenius. Fibres innervated by each branch are intermixed in a boundary region, and the boundary is not related to muscle tendons, muscle fascicle outlines or blood vessels along its length. Therefore, the segregation of innervation in EDL is not the result of a tendinous or connective tissue 'barrier' in the muscle which prevents axons from branching between the two compartments.

Compartmentalization of single motor units has been demonstrated in several muscles (Armstrong, Richmond & Rose, 1982; English & Weeks, 1984; Janun &

English, 1986). The absence of axon reflexes in the present study indicates that each EDL motor axon enters only one nerve branch and that, therefore, single EDL motor units are compartmentalized. Experiments in progress (Balice-Gordon & Thompson, 1986 and in preparation) in which the fibres contained in single EDL motor units have been marked by glycogen depletion confirm that the fibres in single units are confined to one compartment. By selecting which EDL motor units are active, then, the nervous system could potentially activate muscle fibres in only one compartment of the muscle.

Functional role of neuromuscular compartmentalization in EDL

Several hypotheses have been advanced to ascribe a function to compartmentalized innervation in muscle. The demonstration that muscle spindles are preferentially responsive to the activity of motor units within the same compartment (Cameron *et al.* 1981; Binder, 1986) and that I a afferents within a given compartment provide stronger synaptic input to that compartment's motoneurones than to other motoneurones innervating the same muscle has lent support to the idea that the compartmental pattern is related to a localization of the stretch reflex (Hamm *et al.* 1981; Botterman *et al.* 1983; Lucas & Binder, 1984). For example, the compartments within muscles composed of oxidative muscle fibre types typically contain a majority of the muscle's sensory spindles (Botterman *et al.* 1978; Binder, 1986). It is likely that the rat EDL also follows this pattern: the medial portion of the muscle where most of the slow fibres are located also contains the majority of the muscle spindles (Kozeka & Ontell, 1981). This medial region of the muscle is that occupied by K compartment.

English and his colleagues (1982*a*, *b*) have suggested that the segregation of sensory and motor innervation into compartments supplied by branches of muscle nerves might provide a level of control of movement larger than single motor units but smaller than whole muscles. In support of this idea, EMG recordings from various locations within muscles have indicated that selective activation of muscle compartments may exist during certain stereotyped motor outputs (Herring *et al.* 1979; Russell *et al.* 1982; English, 1984). In this regard, we were initially intrigued by the possibility that compartmentalization of EDL might allow for some degree of control of individual foot digits. However, several observations make this possibility unlikely or at the very least not very simple. First, with the exception of the muscle fibres inserting on digit five, which are contained entirely within the F compartment, there is no consistent relationship between the two compartments and the digits of insertion of their muscle fibres. Secondly, although we did not make detailed measurements, we did record the tensions generated at individual tendons of insertion by stimulation of each of the K and F branches. Stimulation of the F branch gave the most tension from digits five and four, whereas stimulation of the K branch gave the most tension from digits three and two. However, appreciable tension was generated at each digit by each nerve branch. Notably, stimulation of the K branch generated tension in the tendon of digit five despite the fact that K does not innervate any fibres inserting on this digit. Thus, there is considerable mechanical coupling between the K and F compartments, probably caused by intramuscular connective tissue as well as the common connective tissue sheath

which wraps the distal tendons. Thirdly, we believe that it is unlikely that EDL is further compartmentalized, for example by further, intramuscular branching of K and F. Glycogen-depletion experiments of single motor units to this muscle (Balice-Gordon & Thompson, 1986 and in preparation), indicate that the fibres of units are located throughout one of the two compartments. While it does not appear likely that the compartmentalization of EDL allows for any fine control of individual digits, there remains the possibility that differential activation of the K and F compartments could be used to control the amount of force produced on lateral *vs.* medial digits and that such control may be important in balance or in locomotion.

How does motor innervation become segregated into compartments?

Perhaps the most parsimonious explanation for the development of the compartmentalized innervation in EDL relies on the segregation of the motor axons which occurs at the point of bifurcation of the muscle nerve. If the K and F nerve branches enter the embryonic muscle in the same disparate locations as in the newborn and adult muscles, then the growth of axons in each branch would be directed into different regions of the muscle. If the motoneurones innervate the first fibres they encounter as they expand from these entry points, the axons in each branch would be expected to capture exclusive territories within the muscle. Convergent innervation would occur wherever the axons growing from each branch met. The extent of this convergent innervation would depend on the speed with which motor axons grow as well as on the speed with which synapses are made and fibres become refractory to further innervation. If, as argued above, the elimination of inputs on these fibres receiving convergent innervation from the two nerve branches were random, then the final appearance of the boundary between the compartments would be related to the initial degree of overlap in innervation.

This explanation of the compartmentalization of EDL attaches particular importance to embryonic nerve branching patterns and the decisions which growing axons make at branch points. These processes have been investigated in the hindlimb of the developing chick by Tosney & Landmesser (1985). They have shown that stereotyped muscle nerve branches form prior to the cleavage of the premuscle mass into individual muscles. Furthermore, the growth cones of axons appear to make binary decisions at these branch points, enter one of the branches, and await the cleavage of the muscle mass before invading each muscle. It is not yet understood how these branching patterns are laid down or how the axons make the choice of which branch to enter. However, in EDL, the binary choice at the K–F bifurcation seems to be influenced by the segmental location of each motoneurone, as the more posterior axons show a preference for the F branch. In addition, the slow–fast identity of the motoneurones may determine their branch choice, because the majority of the slow motoneurones to the muscle must somehow end up within the K branch.

By virtue of the definition of a muscle 'compartment', a nerve branch is associated with each of these muscle regions. The importance of nerve branching in compartmentalizing muscles is also illustrated by the example of a case where an extra, anomalous nerve branch arising during development generates its own unique compartment (Foehring, Sypert & Munson, 1986 and personal communication).

However, the presence of a muscle nerve branch does not always guarantee the presence of a compartment. In some muscles, individual axons undoubtedly bifurcate and enter multiple muscle nerve branches. For example, in the rat soleus, the muscle nerve divides into two prominent intramuscular branches after entering the muscle. As the motor units in this muscle generally have fibres distributed throughout the muscle (Kugelberg, 1973; Thompson, Sutton & Riley, 1984) this muscle is not compartmentalized, and soleus motoneurones probably send axons into both of these branches.

There are probably other muscles where nerve branches segregate motoneurones but none the less the branches do not define compartments. For example, there are rare examples of extraneous extramuscular nerve branches in the rat soleus (Thompson & Jansen, 1977). When present, these branches are found to contain an exclusive portion of the normal motoneurone supply to the muscle. From personal observations of muscle contractions and intracellular recordings of end-plate potentials, these extra nerve branches do not appear to compartmentalize soleus. Therefore, factors in addition to the presence of nerve branches and binary choices by motoneurones probably determine the presence of compartmentalization. Among these factors may be the size of the muscle and the initial exuberance of the motoneurones in forming synapses, the degree of physical separation of the nerve branches and the relative timing of ingrowth of motor axons and formation of muscle fibres.

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