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PROPERTIES OF MOTOR UNITS IN THE TRANSVERSUS ABDOMINIS MUSCLE OF THE GARTER SNAKE

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SUMMARY

1. The organization of motor units in the single-fibre-thick transversus abdominis muscle of the garter snake has been studied. This small segmental muscle (60–100 fibres) contains three distinct fibre types (faster twitch, F; slower twitch, S; and tonic, T) which are predominantly arranged in the repeating pattern F, T, S, T, F, T, etc.

2. Motor-unit maps were obtained by activating an individual motor axon and identifying all of the muscle fibres innervated by that axon, using either the activity-induced uptake of extracellular marker molecules to label presynaptic terminals of the stimulated axon, or systematic intracellular recording to identify muscle fibres activated by the axon.

3. Each muscle contained three types of motor units (F, T and S) that corresponded to the three types of fibres. All of the muscle fibres in a motor unit were of the same type. Each segmental muscle contained approximately the same number of motor units: one to two faster twitch, three to four slower twitch, and three tonic.

4. Each motor unit was dispersed widely throughout the muscle. Fibres within a motor unit were neither clustered nor anticlustered. This suggests that despite the fact that axons are constrained to innervate fibres of the appropriate type, the distribution of each motor unit does not generate the alternating pattern of fibre types.

5. In several experiments, all of the twitch motor units in one segmental component of the muscle were mapped. The projection of any one axon appeared random not only with respect to the muscle's fibre type pattern, but also with respect to the innervation supplied by other axons.

6. Twitch motor units were arranged according to a hierarchy of sizes. In each muscle examined, the largest motor unit was faster twitch; a single faster twitch motor axon usually innervated all of the faster twitch fibres in the muscle (fourteen to twenty-four fibres). This was followed by three to four slower twitch motor units which varied in size from eight to ten fibres to very small motor units containing only four to five fibres.

7. Each tonic motor axon innervated an average of ninety-three end-plates per segmental muscle. The relatively large size of tonic motor units compared to twitch

motor units is related to the ability of tonic muscle fibres to retain polyneuronal innervation into adulthood, both by providing five to seven end-plate sites per fibre, and by allowing terminal boutons from different tonic motor axons to co-innervate the same end-plate. On average, two to three end-plates per tonic muscle fibre were innervated by the same motor axon. There was no tendency for the end-plates of one tonic muscle fibre to be preferentially innervated by a subset of the toxic axons.

8. The disperse and random distribution of motor units in this muscle argues that the repeating pattern of muscle fibre types is of intrinsic rather than neural origin. Consequently, the observed homogeneity of fibre types within one motor unit must depend on precise and highly selective recognition between motor axons and particular muscle fibres, or, alternatively, on a precisely timed ingrowth of different type axons in synchrony with the maturation of different classes of developing muscle fibres.

INTRODUCTION

A motor unit consists of a motoneurone, its peripheral motor axon, and the muscle fibres it innervates. Just as the neuromuscular junction has served as a useful model of synaptic function, the motor unit, because of its accessibility, serves as a good prototype for study of the relationships that exist between neurones and their targets generally. One interesting relationship between motor axons and the muscle fibres they innervate is the correspondence between the phenotype of the motoneurone and that of the innervated muscle fibre (Burke, Levine, Zajac, Tsairis & Engle, 1971; reviewed by Burke, 1981). For example, axons with fast conduction velocities innervate fibres with fast contraction times and vice versa. How such qualitative specificity is established during development is uncertain (reviewed by Kelly, 1983; Kelly & Rubenstein, 1986), but the existence of such 'type matching' in adult animals is not in doubt, primarily because of the sophisticated techniques available for characterizing muscle fibre types within a motor unit (e.g. Barany, 1967; Peter, Barnard, Edgerton, Gillespie & Stempel, 1972; Hoh, 1975; Nemeth, Pette & Vrbova, 1981).

In thinking about how such matching comes about, two alternatives must be considered. One possibility is that the matching only appears secondarily *following* innervation. For example, an axon might innervate fibres more or less by chance but then induces those fibres to differentiate in a way that causes them to obtain common properties. In support of this are a number of observations. Cross-innervation experiments show that the nerve can transform the properties of muscle fibres (see, for example, Buller, Eccles & Eccles, 1960). Furthermore, when the activity pattern of axons is changed experimentally, the muscle fibre properties change concomitantly, demonstrating that motor axons via their activity patterns can cause muscle fibres to express particular contractile properties (see, for example, Salmons & Sreter, 1976). Finally, the clumping of muscle fibres of the same type, not found ordinarily but found in reinnervated muscles, argues for the ability of motor axons to transform the properties of the fibres they innervate (Karparti & Engel, 1968; Kugelberg, Edström & Abbruzzese, 1970).

An alternative explanation for the type matching of muscle and nerve also has much support. In this case, motor axons which are presumed to be intrinsically

different from one another seek out fibres of a corresponding type among a population of heterogeneous fibres. The fact that even in the development of nerveless limbs, muscle fibres differentiate appropriately (Butler, Cosmos & Brierley, 1982; Phillips & Bennett, 1984) indicates that at least primary muscle fibre type differentiation does not depend on innervation. Furthermore, changing the source of innervation by limb transplantation does not affect fibre type differentiation (Laing & Lamb, 1983). The recent demonstration that fibres that are multiply innervated during development are innervated predominantly by motor axons of the same type also argues for some special affinity between particular classes of motor axons and particular types of muscle fibres (Thompson, Sutton & Riley, 1984; see however, Jones, Ridge & Rowlerson, 1987).

Thus the actual cause of the matching between motor axons and muscle fibres during development remains uncertain. Given the strength of the evidence for both intrinsic and extrinsic (neural) influences on muscle fibre properties, it is perhaps most likely that both sets of influences operate to establish the final pattern of motor units and muscle fibre types. We have been studying a muscle in which both intrinsic and neural influences may be analysed directly. This muscle, the transversus abdominis of the garter snake, is a small segmental muscle ($\sim$60–100 muscle fibres) which courses ventrolaterally from a rib of the animal to the ventral mid-line (linea alba). The muscle is unusual in two respects. First, it is but a single fibre in thickness. This allows the position of all fibres to be expressed in terms of a single parameter, the fibre number along the relevant axis (rostral–caudal). Secondly, the muscle contains three distinct fibre types which are arranged in a repeating cellular pattern (Wilkinson & Lichtman, 1985*a*). Moreover, we have developed several techniques which permit complete identification of all the muscle fibres within a motor unit. Thus, the dispositions of various motor units within the muscle may be precisely 'mapped' and compared with each other and with the underlying pattern of fibre types. A preliminary report of some of the findings described has been published (Wilkinson & Lichtman, 1985*b*).

METHODS

Adult garter snakes (*Thamnophis sirtalis*) were cold-anaesthetized in iced water (10 min) and killed by rapid decapitation. Experimental preparations containing three to four segmental components of the thin transversus abdominis muscle were dissected from the animal and placed in reptilian saline solution of composition (mM): NaCl, 145; KCl, 2·5; $CaCl_2$, 3·6; $MgSO_4$, 1·8; KH_2PO_4, 1·0; HEPES, 5·0; glucose, 3·0; NaOH as needed for pH 7·4. Several contiguous segments of this muscle were exposed from the ventral side and removed, along with their origins on the ribs and mid-line insertions, at the linea alba (see Wilkinson & Lichtman, 1985*a*, for details of dissection procedure).

Electrophysiology. Experiments were performed at room temperature ($\sim$25 °C) on the stage of an inverted microscope equipped with interference contrast (Nomarski) optics. The transversus abdominis muscle nerve was cut near its branching point from the ventral segmental nerve trunk and cleaned of connective tissue for a distance of 2–3 mm. The nerve was then placed on a chlorided silver wire and drawn into a thin polyethylene tube containing paraffin oil. A spiral of silver wire wrapped around the tube served as a reference. This 'hook-in-oil' electrode was used for recording antidromically propagated action potentials initiated at nerve terminals (see below) and, occasionally, for stimulation of the muscle nerve.

Intracellular recording from muscle fibres was performed using micropipettes filled with

1 M-sodium acetate (resistance, 80–120 MΩ). Postsynaptic responses to axon stimulation were usually recorded without the addition of paralysing agents to the bath. Contractile responses (of individual motor units) to single stimuli were too weak to dislodge the recording pipette in tonic and slower twitch motor units. The more powerful faster twitch units often dislodged the pipette (after their action potential was recorded), but these large, low-resistance fibres (Wilkinson & Lichtman, 1985*a*) were not easily damaged and could be reimpaled if necessary.

Tension developed by motor units was measured with a commercial transducer (Model 407, Cambridge Technology, Cambridge, MA). The transducer was attached via a small hook to the centre of the tendinous insertion (linea alba), while the rib end of the muscle was held fixed by magnetic pins. Because of the muscle's thinness, its 'resting length' in isolation was quite short, being determined primarily by the elasticity of underlying connective tissue. For this reason, a fixed incremental increase beyond this length (i.e. 110%) was not a suitable criterion for adjusting length prior to tension measurements. Instead, the length of each preparation was adjusted for maximum tension upon supramaximal stimulation of its nerve. A computer (980B, Texas Instruments, Dallas, TX) was used to digitize and store tension records.

Motor units were activated by extracellular stimulation of a single nerve terminal (or preterminal axon branch) located with Nomarski optics (Cliff & Ridge, 1973; see Plate 1*A*). A glass pipette containing 150 mM-NaCl (tip diameter, 15–25 μm) was used to deliver 10–100 V, 200 μs rectangular voltage pulses of either polarity (whichever had the lowest threshold); an annulus of silver foil in the bath was the return electrode. With this type of stimulation, an action potential initiated at a nerve terminal propagated to invade all other terminals of the same axon (see below). In addition, the action potential entered the muscle nerve (propagating antidromically) from which it was extracellularly recorded to verify activation of the motor unit. Generally, the action potential recorded from a particular unit was unique in shape, amplitude and latency, and thus served as a signature for that axon's activation. Spontaneous afferent activity from muscle spindles, which was always present on the extracellular nerve recordings, provided a convenient reference for comparison: action potentials from twitch axons were similar to those from primary sensory axons, while action potentials from the more slowly conducting tonic axons were smaller and of longer duration.

Activation of exactly one motor unit was confirmed by the presence of a non-fractionable action potential in the muscle nerve together with a non-fractionable tension response following suprathreshold stimulation of a nerve terminal (Fig. 1 and Plate 1*A*). Evidence that all muscle fibres within a motor unit were indeed activated by stimulation of one terminal (i.e. that no conduction failure occurred at branch points) was as follows. (1) No variability in tension responses to repeated stimuli was seen, which would have indicated occasional branch failure. Moreover, end-plate potentials recorded from several muscle fibres, each innervated by an axon stimulated from a nerve terminal, exhibited no 'failures', even in response to rapidly repeated stimulus trains (50–80/s). (2) The same motor unit activated from any of its nerve terminals produced the same tension response. Although the temporal sequence in which the motor unit's fibres were activated probably varied by a few tenths of a millisecond, depending on which terminal was stimulated, this effect was evidentially too small to be seen on the time-scale of tension records. (3) One motor unit in each preparation (that with the lowest threshold) could be activated in isolation orthodromically from the muscle nerve by gradually increasing stimulus strength until the initial minimum tension response was seen. Activation of this same unit antidromically from an end-plate then produced an identical tension response. (4) In preparations where systematic intracellular recording was performed from all twitch fibres in one muscle, no fibre was found which could not be activated from one of four to five nerve terminals, each terminal belonging to a different twitch motor unit.

Anatomy. Motor units were identified for 'mapping' by selectively labelling all terminals of one axon. This was accomplished by stimulating the axon (from one terminal visualized with Nomarski optics; see above) in the presence of one of several extracellular probes which are internalized exclusively by active nerve terminals. Two types of probes were used, horseradish peroxidase (HRP, type VI, Sigma Chemicals, St Louis, MO), and certain sulphonic acid derivatives of low molecular weight fluorescent molecules (sulforhodamine 101, red fluorescing; fluorescein 5,6 sulphonic acid, green; 8-hydroxypyrene trisulphonic acid, blue; Molecular Probes, Eugene, OR; see also Lichtman, Wilkinson & Rich, 1985). The probes are presumably taken up as a consequence of vesicle recycling which occurs at the membranes of active terminals (Heuser & Reese, 1973).

After fixation, labelled terminals (and thus the muscle fibres which underlay them) could be identified in the light microscope. The methods developed for use of HRP or the fluorescent probes were fundamentally the same, but differed in detail as described below. In general, the HRP technique was more time-consuming but produced permanently stained preparations. In contrast, the fluorescent probes were faster and easier to work with, but the fluorescence faded with time (apparently by leakage of the terminals) so that preparations had to be examined either while living or soon after fixation (within 24 h).

For HRP labelling, the preparation was placed in a Ringer solution bath and a nerve terminal was selected for stimulation. After confirming activation of a single motor unit (see above), curare (200 μg/ml) and HRP (20–40 mg/ml) were added to the bath, thereby preventing motion of the stimulated terminal relative to the pipette during the uptake procedure. The axon was stimulated repeatedly for a total of 15 min by a train of 200 μs pulses (40–100/s for 5 min, 2 min rest; repeated three times). During the stimulation, the action potential was continuously monitored to ensure that the chosen unit (and only that unit) was active. Often, the muscle fibre underlying the stimulated terminal would contract (by direct stimulation), necessitating a slight readjustment of the stimulus pipette. In a few instances, the movement was troublesome and was therefore arrested by purposely damaging the fibre. This was done by momentarily moving the stimulus pipette away from the end-plate, touching the fibre with the pipette, and passing a large direct current for several seconds. After completion of the stimulus procedure, the preparation was rinsed for a total of 30 min in five washes of cold, agitated low-Ca^{2+} (0 mM-$CaCl_2$) high-Mg^{2+} (5·1 mM-$MgSO_4$) Ringer solution for 1 h to reduce background staining with a minimum of vesicle recycling. The best light-microscopic visualization of HRP-filled terminals was obtained using the silver-intensified diaminobenzidine procedure of Gallyas, Görcs & Merchenthaler (1982). Preparations were counter-stained for acetylcholinesterase (Bondi & Chiarandini, 1980) so that end-plates could be located quickly and scored for the presence or absence of HRP reaction product. The cholinesterase staining procedure was performed as an additional step by inserting it into the procedure of Gallyas *et al.* (1982), after the reaction with diaminobenzidine but before silver-intensification of the reaction product.

In contrast to HRP, labelling of motor units by the activity-dependent uptake of fluorescent probes required only 3–5 min of stimulation with one of the probes applied to the bath at a concentration of 100–200 μg/ml. The muscle nerve was supramaximally stimulated at a frequency of 20–50 Hz (3 s on, 2 s off). The preparation was then washed (~six changes of cold Ringer solution) for a total of 30 min on a rotating shaker in the dark. In this way all of the nerve terminals were labelled within one segment of the muscle to provide a background stain (analogous to the use of cholinesterase staining in the HRP preparations). The nerve terminals within a single motor unit in the same muscle were then labelled selectively by covering the muscle with a solution containing one of the other fluorescent probes. An end-plate was located using an inverted Nomarski microscope and stimulated with a continuous train of pulses (200 μs; 20–50 Hz for 3–5 min). Steps taken to ensure that only one motor unit was stimulated were the same as described above for HRP labelling. The preparation was then washed in low-Ca^{2+}, high-Mg^{2+} Ringer solution for 30 min, fixed in 4% paraformaldehyde for 30 min, and mounted on a slide in *p*-phenylenediamine phosphate-buffered saline–glycerine solution (Johnson & de C. Nogueira Araujo, 1981) to prevent fading. This second stimulation of a single motor unit caused the partial depletion of the first dye, presumably due to continued vesicle recycling of the restimulated terminals as they became filled with the second probe. By this procedure, terminals of one motor axon were labelled with one colour probe, which all the remaining nerve terminals were labelled with another colour.

RESULTS

Innervation pattern of twitch motor units

Because the transversus abdominis displays a unique alternation of twitch and tonic muscle fibre types (Wilkinson & Lichtman, 1985*a*), it was of interest to examine how the innervation patterns of individual motor axons related to the alternation. The dispositions of nine twitch-type motor units (nine muscles, seven snakes) were determined using the activity-induced uptake technique (see Methods). After

locating the end-plate of an arbitrarily chosen twitch muscle fibre using Nomarski optics, the motor axon innervating the end-plate was stimulated extracellularly in the presence of HRP. A stimulus pipette was positioned near an end-plate (Plate 1*A*); voltage applied to the pipette resulted in a non-fractionable nerve action potential in the proximal nerve and muscle tension from one motor unit (Fig. 1)

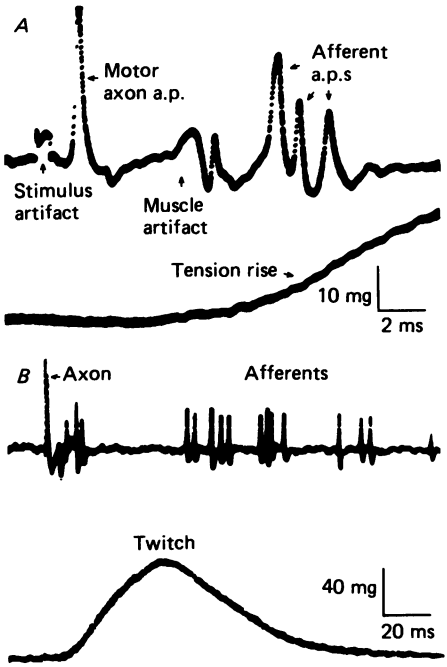

Fig. 1. Method for stimulating a single motor unit from one of its nerve terminals. *A*, activation of exactly one axon (in this case twitch) was confirmed by the presence of a non-fractionable action potential (a.p.) in the muscle nerve (upper trace) together with a non-fractionable tension response (lower trace). Afferent action potentials initiated by muscle spindles occurred with long latency, signalling the onset of contraction. *B*, similar records as in *A*, but on a longer time-scale to reveal the entire tension response.

which were taken as evidence that only a single axon was active and all of the fibres in that motor unit were activated (see Methods). After stimulation, the muscles were stained for cholinesterase and HRP reaction product, fixed, and whole-mounted on standard microscope slides. Motor unit 'maps' were then constructed. First, each fibre in the muscle was classified as either twitch or tonic according to its size and surface appearance, and the shape, size and number of its end-plates (see Wilkinson & Lichtman, 1985*a*). Next, each end-plate was scored for the presence of HRP, which indicated that it belonged to the stimulated motor unit (Plate 1*B*). A representative map is shown in Fig. 2. The position of the axons comprising the end-plate bands is drawn approximately; however, whether the end-plate is above or below the nerve and the sequence of twitch and tonic muscle fibres, including those within the motor unit (white circles), is precise. Intrafusal muscle fibres, associated with muscle spindles, are known to receive collateral innervation from motor axons in the snake (Cliff & Ridge, 1973), but were not studied.

Results (Fig. 3) indicated that twitch motor units varied in size, the average containing about ten fibres. Each of the motor units appeared to be dispersed more or less randomly throughout the muscle, as can be seen by examining Fig. 3 (each horizontal diagram is one muscle; motor units are denoted by white circles). To confirm this, the spatial disposition of fibres within each labelled motor unit was

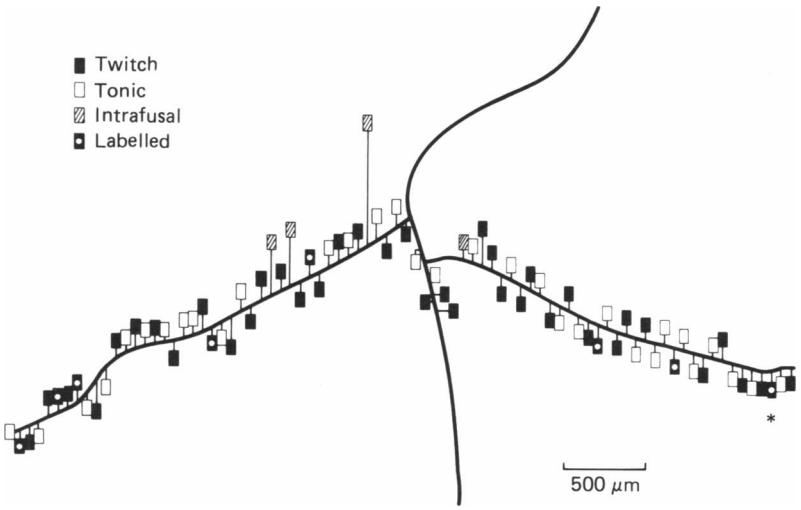

Fig. 2. Typical twitch motor-unit map, showing location of the nerve entering the muscle from the rib (above), all the nerve terminals in the major end-plate band (rectangular boxes) and the type of muscle fibre innervated by each nerve terminal. Muscle fibres (not shown) are oriented vertically, coursing from a rib (above) to the linea alba (below; rostral is to the left). Fibre types (twitch or tonic) were determined by surface appearance, diameter and the shape of the end-plate site as revealed by cholinesterase staining. Twitch fibres each possessed one large oval- or spade-shaped end-plate whereas tonic fibres were innervated at multiple end-plate sites (only one of which is shown), each containing fewer and more linearly arranged synaptic boutons (see Wilkinson & Lichtman, 1985*a*, for complete description of fibre types). One motor axon was activated from an end-plate (*) in the presence of horseradish peroxidase, thereby labelling its terminals (see Plate 1*B*). Fibres underlying the labelled terminals and therefore belonging to the activated motor unit are indicated by white circles. Intrafusal fibres (associated with muscle spindles) were mapped in some experiments; the location of their sensory capsule is indicated by striped symbols.

tested statistically, using a appropriate χ^2 test for small expectations (adapted from Nass, 1959). The tests showed that each motor unit was indeed dispersed throughout the muscle in a manner not significantly different from random ($P > 0{\cdot}1$, double-tailed); thus, motor units were neither clustered in one or more regions of the muscle nor were they 'anticlustered', i.e. distributed with a more uniform spacing than would be predicted by random innervation of fibres. However, without exception, all nerve terminals in identified twitch motor units were of the twitch type and, by appearance, all of the fibres they innervated were also of the twitch type. Thus, twitch motor axons had strict preference for twitch muscle fibres, without regard for the fibres' position within the muscle.

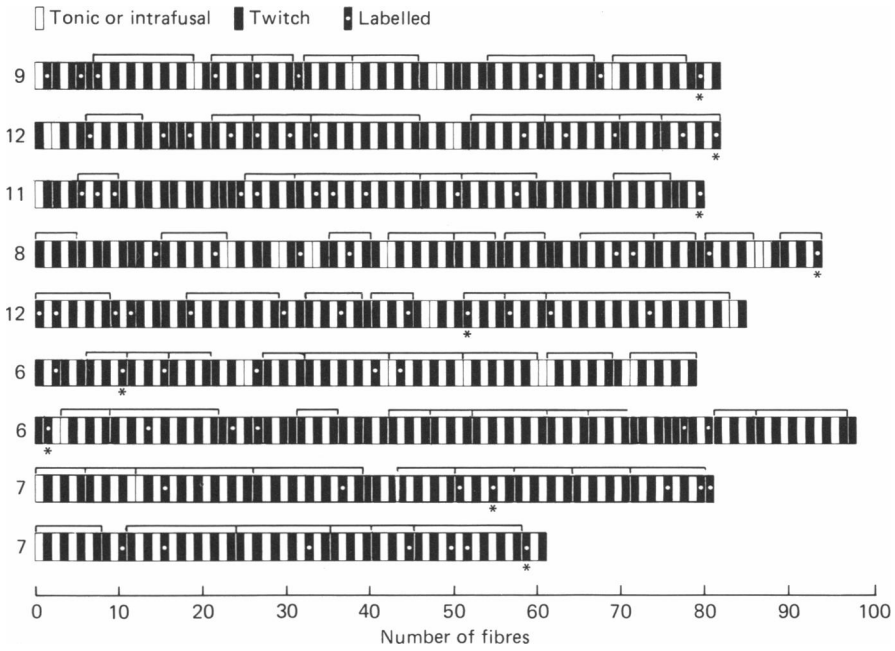

Fig. 3. Summary of nine twitch motor units mapped according to the method of Fig. 2. Each horizontal diagram ('piano keys') represents one muscle in which one axon was activated from a twitch fibre end-plate (asterisk) in the presence of horseradish peroxidase. Muscle fibres underlying labelled nerve terminals (white circles; total number indicated at left) were all of the twitch type but were otherwise randomly distributed in the muscle, showing no relation to the regular alternation of twitch (black) and tonic (white) muscle fibres. Regions of strict fibre type alternation are indicated by horizontal brackets above the diagrams.

Characteristics of twitch motor unit subtypes

Two twitch muscle fibre subtypes are present in the transversus abdominis (Wilkinson & Lichtman, 1985*a*), similar to those in other garter snake muscles (e.g. Ridge, 1971). The two types differ in surface appearance when viewed in living preparations (rough *vs.* smooth; see Wilkinson & Lichtman, 1985*a*), but they differ only slightly in contraction time, unlike, for example, mammalian fast and slow twitch types. We have therefore chosen the terminology 'slower twitch' for the rough-surfaced fibres and 'faster twitch' for the smooth fibres, to avoid confusion with mammalian fibre types. In addition, the twitch subtypes differ in their microchemical assay levels of certain energy-dependent enzymes (R. S. Wilkinson & P. M. Nemeth, unpublished). The microchemical assays show that the faster twitch fibres are primarily glycolytic and therefore resemble mammalian type IIB fibres (fast twitch, fatigable) while the slower twitch fibres are oxidative–glycolytic and resemble mammalian type IIA fibres (fast twitch, fatigue-resistant; P. M. Nemeth, personal communication). Thus, the two twitch fibre types in the transversus abdominis are probably both fast twitch subtypes in mammalian terminology.

To study the disposition of motor units belonging to each twitch subtype, a map

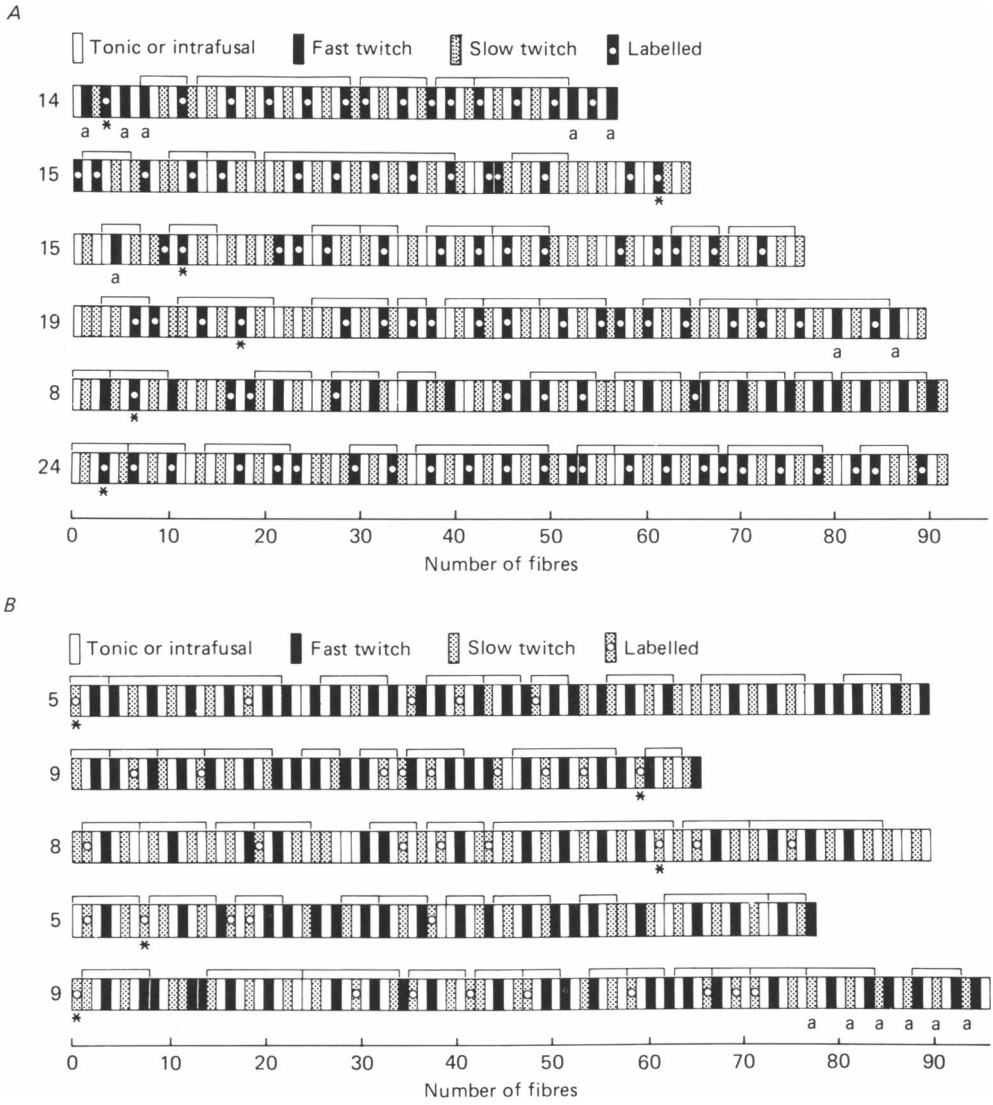

Fig. 4. Twitch motor units in the transversus abdominis identified according to twitch subtype. Each horizontal diagram represents one muscle. Living fibres were classified as tonic (T), faster twitch (F), or slower twitch (S) according to surface appearance and end-plate morphology. A terminal innervating a faster twitch fibre (*A*) or slower twitch fibre (*B*) was stimulated in the presence of an activity-dependent fluorescent probe (selected fibre marked by asterisk). The fibres underlying labelled terminals (marked by white circles) were all of the same twitch subtype as the one whose end-plate was stimulated (number of innervated fibres in each labelled motor unit is shown at left). Faster twitch units (*A*) were generally larger than slower twitch (*B*) and usually contained all faster twitch fibres in the muscle, excepting those innervated by the adjacent segmental nerve (indicated by a). Regions of the muscle displaying both the primary alternation of twitch and tonic fibres and the secondary alternation of twitch subtypes (i.e. F, T, S, T, etc.) are indicated by brackets above diagrams).

indicating the location of all fibres within the muscle was constructed by noting with Nomarski optics the surface appearance and position of living fibres. After identifying the type of each muscle fibre, the green fluorescent probe was added to the bath and the muscle nerve stimulated supramaximally for 5 min (see Methods). This filled all nerve terminals in the muscle with the green probe. The preparation was then rinsed in Ringer solution, and a twitch nerve terminal was selected for stimulation ($\sim$5 min) in the presence of the red fluorescent probe to partially deplete the green probe and fill the terminals with the red probe. Thus, one motor unit's terminals were made to fluoresce red while all other terminals in the muscle fluoresced green. After fixation, muscle fibres underlying red terminals and therefore belonging to the labelled motor unit were identified according to their position within the previously constructed map.

Results of eleven such experiments (Fig. 4) showed that all fibres innervated by one twitch axon were of the same twitch subtype. The brackets above the diagrams in Fig. 4 show regions of the muscles displaying both the primary fibre type alternation (twitch, tonic, twitch, tonic, etc.) and the secondary alternation of twitch subtypes (faster twitch, tonic, slower twitch, tonic, etc.). Consistent with results (Fig. 3) from experiments in which twitch motor units were studied without regard to subtype, slower twitch units appeared to be randomly dispersed throughout the muscle, in such a way that the projection of one motor axon bore no correlation to the underlying pattern of fibre types (Fig. 4*B*). Faster twitch motor units, in contrast, were often large enough to include all faster twitch fibres in the muscle (five of six experiments; Fig. 4*A*, see also Plate 2). As a result, one motor axon's projection and the location of faster twitch fibres within the alternating pattern were often identical.

Interrelationship of twitch motor units

As described above, many of the maps of individual twitch motor units within the transversus abdominis (Figs 3 and 4) showed no relation between the projection of one axon and the underlying pattern of fibre types, even in localized regions of the muscle. To see how motor units were arranged relative to each other required that two or more motor units be mapped in the same muscle. Because the muscle contains four to six faster and slower twitch motor units (as evident from the four to six tension steps in response to graded nerve stimulation) and we had only three differently coloured activity-dependent probes, the motor units were mapped by the more laborious method of systematic intracellular recording from each of the muscle fibres (about eighty) in the muscle.

After first mapping the position and type (based on surface appearance) of all fibres in the muscle, a twitch nerve terminal was selected for stimulation. Single stimuli were delivered to the terminal; the twitch tension and muscle nerve action potential were monitored to assure that exactly one motor unit was active (see Methods). Next, each twitch fibre in the muscle was impaled near its end-plate and tested for a postsynaptic response. Presence of an action potential was taken as evidence that a particular fibre belonged to the motor unit being stimulated (see Methods). When all such fibres were located, a second nerve terminal was stimulated, and recordings were made from the remaining twitch fibres. The process was

repeated until nearly all twitch fibres in the muscle were accounted for. A few fibres near one or both edges of the muscle typically remained unidentified and were assumed to be innervated by the adjacent segmented nerve, as occurs frequently in this muscle (see Fig. 4 and Lichtman *et al.* 1985). This could usually be confirmed by direct visualization of axons invading the muscle from the adjacent segment and giving off terminals on the fibres. In one preparation where the segmental nerves

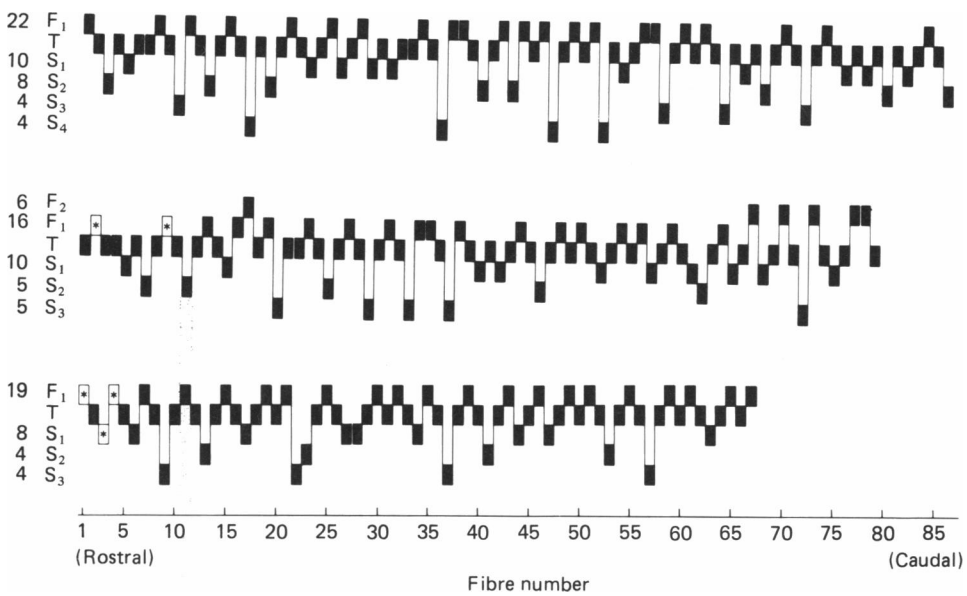

Fig. 5. Location of all twitch motor units in one transversus abdominis muscle. Three muscles from three snakes are shown. Motor units were mapped by sequential stimulation of motor axons and systematic intracellular recording to locate activated muscle fibres. Fibres of all types are represented by black bars. The horizontal position of each bar represents that fibre's position within the muscle, as in Figs 3 and 4. The bar's vertical position indicates to which motor unit the fibre belonged (labelled at left according to size; F, faster twitch; S, slower twitch; T, tonic; number of fibres innervated shown at far left; subscripts define different motor units of the same type). In each muscle, a type F motor unit was the largest, followed by type S units of intermediate and small size. Tonic fibres were identified but not studied. Asterisks identify fibres innervated by adjacent segmental nerve.

anastamosed (so that the segmental origin of particular terminals could not be determined by visual inspection), the adjacent segmental nerve was stimulated while recording from the fibres in question. This procedure confirmed that the fibres were innervated from the adjacent muscle.

Results of three experiments (three snakes) in which all twitch motor units were mapped are shown in Fig. 5. Each diagram represents one muscle. Position of fibres within the muscle is represented horizontally, as in Figs 3 and 4, while the vertical displacement of the bars representing each muscle fibre indicates to which motor unit the fibre belonged. Tonic muscle fibres are shown, but were not classified into individual motor units (see below). As in single-motor-unit experiments, axons of all three types innervated exclusively muscle fibres of the corresponding type, but did

so in a random manner with respect to the fibres' position in the muscle. Furthermore, the motor units appeared to be randomly intermingled.

In the three muscles in which all twitch motor units were mapped, the motor units were arranged in a stereotyped hierarchy of sizes. The largest motor unit was faster

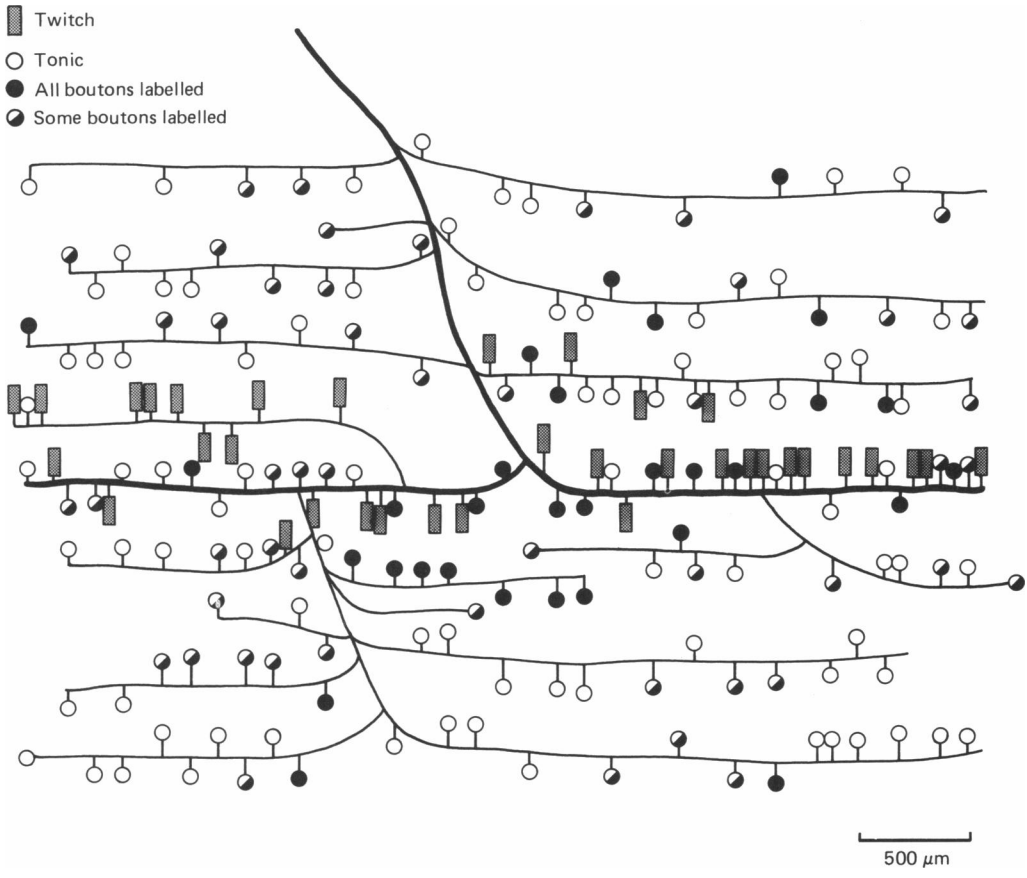

Fig. 6. The projection of one tonic motor axon in the transversus abdominis. Muscle fibres course vertically as in Fig. 2. The muscle nerve branches to form the main end-plate band (heavy lines) as well as several tonic end-plate bands spaced at about 1 mm intervals along the muscle's length. All end-plates in the muscle were made visible (using the green activity-dependent probe) and identified as tonic (circles) or twitch (stippled rectangles). Terminals of one tonic axon were filled in addition with the red probe. End-plates exclusively innervated by red-labelled terminals are shown as all-black. End-plates partially innervated (i.e. shared with one to two other axons) are shown as half-black. Note the wide projection of the axon, into virtually all of the end-plate bands. Only tonic muscle fibre end-plates were innervated.

twitch, and, consistent with the experiments shown in Fig. 4*A*, this axon innervated all or nearly all faster twitch fibres in the muscle (average, nineteen fibres). Next were one to two intermediately sized slower twitch motor units (average, nine fibres each), followed by two small slow twitch units, each of which contained only four to five fibres.

Innervation patterns of tonic motor units

Tonic muscle fibres in the snake (and other species) lack action potentials (see Lichtman *et al.* 1985) under ordinary conditions and therefore contract in direct response to postsynaptic potentials (reviewed by Morgan & Proske, 1984*a*). The lack of propagated action potentials is compensated for, in part, by multiple end-plate sites, which depolarize the fibres from several foci along their length. Snake tonic fibres are also known to be polyneuronally innervated at many individual end-plates 'shared' by two or three innervating axons (Lichtman *et al.* 1985). Thus, tonic motor units overlap, in that individual muscle cells (and even end-plate sites) can belong to more than one motor unit.

Tonic motor unit maps were constructed in essentially the same way as those of twitch units. A single tonic axon was stimulated in the presence of the red activity-dependent probe, after first labelling all nerve terminals in the muscle with the green (background) probe. Background labelling was performed by stimulating both the muscle nerve which contained the tonic axon under study and also the muscle nerves supplying the adjacent rostral and caudal segments. This ensured that all terminals in the muscle under study would be labelled with the background probe, including some terminals supplied by axons anastamosing from the adjacent segments (see Lichtman *et al.* 1985).

Exactly four types of labelled nerve terminals were seen in all such experiments (Plate 1*C–F*). Large twitch-style terminals (Plate 1*C*) were filled exclusively with the green background probe. Thus, no innervation of twitch-type end-plates by the stimulated tonic axon (as would have been evident from the presence of one or more red boutons) was observed. End-plates identified as tonic were either not innervated by the stimulated axon (all boutons green; Plate 1*D*), were partially innervated by the stimulated axon (some boutons red, some boutons green; Plate 1*E*), or were exclusively innervated by stimulated axons (all boutons filled with the red probe; Plate 1*F*). To construct maps of tonic motor units, each tonic fibre end-plate in the muscle was located with fluorescence microscopy for the background probe, and its position within the muscle noted. The end-plate was then scored, according to the criteria described above, as being not in the unit (all boutons green), exclusively in the unit (all boutons red), or shared (mixed population of red and green boutons).

Each tonic axon was found to be distributed widely, not only in the sense of innervating fibres throughout the muscle's width (similar to twitch axons), but by coursing in all of the five to eight tonic end-plate bands which are located at approximately 1 mm intervals along the muscle's length (Wilkinson & Lichtman, 1985*a*). One tonic motor unit map is shown in Fig. 6. This axon innervated about half (80 out of 164) of the tonic muscle fibre end-plates in the muscle. Of the 80 innervated end-plates, 39% (31 out of 80) were innervated exclusively by this axon (filled circles); the rest were innervated by more than one tonic axon (half-filled circles). The results were generally the same for each of the three tonic motor units mapped. On average, 47% of the tonic end-plates were innervated by the tonic axon being studied, and 34% of these were singly innervated by that axon. These results allowed us to estimate the total number of tonic axons within each muscle. If each axon on average singly innervates 34% of the end-plates it contacts, and it contacts 47% of

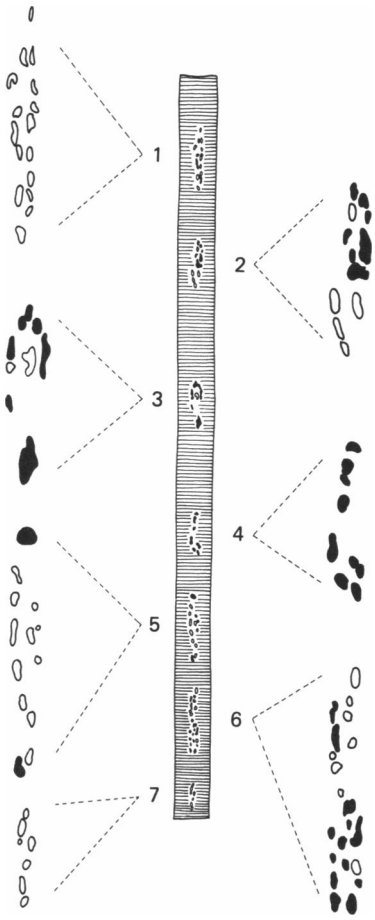

Fig. 7. One axon's contribution to the innervation of a tonic muscle fibre. All terminals in the muscle were filled with the green probe, while those of one tonic motor axon were filled in addition with the red probe (see legend for Plate 1*C–F*). This tonic fibre, shown schematically, had seven end-plate sites. Camera lucida drawings indicate arrangement of terminal boutons at each end-plate (revealed by the green probe). Boutons of the axon under study (red-fluorescing) are shown as black; boutons not belonging to that axon are shown as white. End-plate 4 was solely innervated by the axon; end-plates 1 and 7 were not innervated by that axon. Statistically, the pattern of innervation supplying end-plates of one tonic fibre was the same as that supplying all tonic end-plates in the muscle, suggesting little tendency of motor axons to choose one tonic muscle fibre over any other.

the tonic fibre end-plates in the muscle, then 16% of the muscle's tonic end-plates are singly innervated by each tonic axon. From previous intracellular recording we had found that 51% of tonic fibre end-plates were multiply innervated (Lichtman *et al.* 1985). Thus, if each axon is the exclusive source of innervation for 16% of the end-plates, then three such axons would innervate 48% of the fibres singly, leaving the remaining 52% multiply innervated. Because 51% of the end-plates are, on average, multiply innervated, we estimate that there are three tonic axons per muscle. Additional evidence for this number is that exactly three axons were visualized in tonic end-plate bands with Nomarski optics, and exactly three action potentials

could be recruited (and seen by recording from the muscle nerve) upon gradual increase of stimulus strength delivered to a tonic end-plate band. Because we have not studied more than one tonic axon per muscle, it is not known whether a systematic hierarchy in tonic motor unit size exists (analogous to the hierarchy of twitch unit sizes; Fig. 5). However, the fact that each of three tonic units mapped in different muscles was approximately the same size (44%, 44% and 49% of tonic end-plates innervated) argues that no such hierarchy exists.

The muscle shown in Fig. 6 contained 36 tonic muscle fibres and 164 tonic end-plates, meaning that each fibre had about five end-plate sites. If an axon distributed its innervation randomly to tonic fibre end-plates, 47% or two to three per muscle fibre should be innervated by the labelled axon. Alternatively, end-plates on one muscle fibre may be preferentially innervated by the same axon, as has been documented for twitch muscle fibres in the frog (Nudell & Grinnell, 1983). Thus, despite the apparent overall randomness of one tonic axon's projection (Fig. 6) it remained possible that some specificity of innervation existed at the level of single cells, i.e. an axon might strongly innervate one fibre, say at all of its end-plates, while weakly innervating another.

To examine innervation of individual tonic muscle fibres in detail, one tonic motor unit was labelled with the red activity-dependent probe, while all the remaining terminals in the muscle were labelled with the green activity-dependent probe, as described above. The muscle was stretched laterally so that each small tonic fibre could be followed more easily along its length to ensure unambiguous assignment of end-plates to each fibre. Innervation of a typical fibre is shown in Fig. 7. We found that each tonic muscle fibre had end-plates which were singly, multiply, or not innervated by the axon under study, suggesting no strong affinity between particular tonic axons and particular tonic muscle fibres. To measure more precisely the strength of innervation from the labelled axon, for each end-plate the total number of terminal boutons (green and red) were counted, and the subset of boutons supplied by the stimulated axon (red) were counted. On fourteen muscle fibres studied (two muscles), the stimulated axon supplied on average 27% of the boutons, which is the same as the percentage of boutons supplied by one tonic axon as previously obtained for all the end-plates in a muscle (Lichtman *et al.* 1985). The percentages of boutons on each fibre supplied by the separately labelled axon were 15, 16, 18, 21, 22, 23, 26, 26, 26, 36, 36, 44 and 53. These values are approximately normally distributed about their mean (27% in each muscle studied), suggesting that the fibres studied belonged to a single population. Thus, no evidence was found to suggest that fibres are preferentially innervated by particular axons, which would have been indicated by a bimodal distribution, with fibres either strongly or weakly innervated relative to the mean (see Discussion).

DISCUSSION

Projection of motor axons

Motor unit maps indicated that axons of all three types (faster twitch, slower twitch, and tonic) projected widely and randomly throughout the muscle. Even when several motor units in one muscle were mapped, no discernible pattern of innervation, potentially from axons acting in concert, was detected. Randomness

was demonstrated most clearly among the slower twitch units. This was because the large faster twitch and tonic motor axons often innervated all the correspondingly typed fibres in the muscle. Although consistent with random innervation, these maximally sized projections do nothing to prove it. However, tonic axons did innervate individual tonic *end-plates* in an apparently random manner (about half in each of five to seven end-plate bands; see below).

In marked contrast, the disposition of muscle fibres when classified according to type, but irrespective of motor unit, was not random. The three fibre types (slower twitch, S; faster twitch, F; and tonic, T) alternated in a regular pattern (F, T, S, T, etc.; Figs 3 and 4). This alternation occurs in this muscle at a high statistical confidence level ($P < 0{\cdot}001$; Wilkinson & Lichtman, 1985*a*).

The observed randomness of axonal projections rules out any pronounced spatial pattern or territorial preference for axons. There was, however, remarkable specificity with respect to muscle fibre *type*. One class of axons elaborated exclusively tonic-style nerve terminals and innervated exclusively tonic muscle fibres. Similarly, the two classes of twitch axons elaborated only twitch-style terminals; the terminals were found to innervate either all slower twitch or all faster twitch fibres. This 'type matching' of motoneurone to muscle fibre was absolutely precise for all three classes of motor units, based on observation of all terminals supplied by an axon in one muscle.

The simultaneous existence of regular fibre type alternation, random spatial projection of axons, and precise type matching limits to two the types of hypotheses which might explain the origin of fibre-type heterogeneity in this muscle (see also Wilkinson & Lichtman, 1985*a*), as follows. (1) Adult muscle fibres differentiate through the execution of an intrinsic genetic program (e.g. Butler *et al.* 1982). Type matching is achieved through neurone-target recognition, perhaps chemically mediated. (2) Ingrowth of a particular class of motor axons occurs synchronously with the 'maturation' of a corresponding class of muscle fibres (Rubenstein & Kelly, 1981), in such a way that only mature fibres are receptive to innervation. These two hypotheses need not be mutually exclusive. For example, tonic fibres might mature and become innervated first, followed by a period wherein each of the twitch-fibre subtypes is appropriately innervated by means of specific recognition. Both hypotheses are similar, though, in that they imply an intrinsic, or myogenic (as opposed to neurogenic), origin for fibre-type diversity in this muscle. However, it should be noted that only two properties of the fibres need have this intrinsic origin: their organization into a regular pattern of types (which may reflect their lineage history, see Wilkinson & Lichtman, 1985*a*), and either some (presumed chemical) factor which permits their recognition by axons or a factor which determines their maturation time. All other properties which differ among adult types (e.g. myosin isozyme profile, contraction time, energy-dependent enzyme activities; reviewed by Kelly & Rubenstein, 1986) might well be induced by the innervating axons. Thus, the abundant evidence from reinnervation studies, which attributes certain properties of muscle fibres to the innervating motoneurone, is not inconsistent with the present results.

Size distribution of motor units

The accessibility of the transversus abdominis preparation provides an opportunity to examine the disposition of all motor units in one muscle. The size of projection (number of fibres innervated) for each of the seven to nine motor axons in the muscle was stereotyped. In three muscles where all twitch motor units were studied, two slower twitch axons per muscle innervated four to five fibres (the smallest projections). Next in size were one or two slower twitch axons innervating eight to ten fibres each. Next, one faster twitch axon usually innervated all of the eighteen to twenty-four faster twitch fibres per muscle. Each of three tonic axons probably innervated all of the thirty-five to fifty tonic muscle fibres per muscle, the largest projections. Thus, the muscle contains, on average, four groups of motor units in terms of size, each group comprising one to three units.

The fact that slower twitch motor units are smaller than those comprising faster twitch fibres is consistent with one aspect of the size principle (Henneman, 1957), an inverse relation between motor unit size and contraction time (see Enoka & Stuart, 1984). Furthermore, we have found that, within one muscle, contraction times vary inversely with motor unit size not only between faster and slower twitch but also among different slower twitch units (R. S. Wilkinson & M. N. Faddis, work in progress). This relation has been reported for another snake muscle (Cliff & Ridge, 1973; Hammond & Ridge, 1978). In contrast, tonic motor units have by far the slowest contraction times (the same as single tonic fibre contraction times, see Wilkinson & Lichtman, 1985*a*), yet they contain the largest number of fibres. One explanation for this discrepancy from the size principle is that the maximum force produced by tonic muscle fibres is smaller than that of twitch fibres (Wilkinson & Lichtman, 1985*a*) and, furthermore, a tonic fibre is probably not completely activated by a single tonic axon (see Morgan & Proske, 1984*b*). Thus, in terms of force generated (a functional indicator of motor unit size), the effective size of tonic motor units is much smaller than their fibre numbers indicate.

Quantitative specificity

Two types of neural specificity are apparent in the transversus abdominis muscle. As discussed above, precise qualitative matching exists between motor axon and muscle fibres according to type. Also, quantitative matching of target size (number of fibres) among different axons occurs, giving rise to the observed stereotyped distribution of motor unit sizes. The question arises as to whether the two types of specificity are independent or represent two aspects of the same developmental or regulatory mechanisms. This question has been difficult to address in twitch muscles because the two types of specificity seem to go hand-in-hand. Motor unit size is inversely correlated with contraction time, and the latter is related to muscle cell phenotype. Thus, because each twitch fibre is singly innervated, the qualitative (e.g. contraction time) and quantitative (motor unit size) aspects of each axon's projection are simultaneously represented by one unique group of target muscle fibres.

This problem is potentially avoided by examining the projections of tonic motor axons in the transversus abdominis, which share the same population of target cells

by virtue of extensive polyneuronal innervation. Tonic axons coursed throughout the muscle, branching into all available end-plate bands. Thus they had access to virtually all ~200 tonic end-plates in the muscle. Furthermore, each of the five to seven end-plates on one tonic fibre seemed to be an independent target; an axon's strength of innervation at one end-plate (as indicated by number of boutons) did not predict its strength of innervation at the fibre's other end-plates. In other words, each tonic fibre was shared, about equally, by all three tonic motor axons. The finding that each axon innervated only a fraction (about 47%) of the available end-plates therefore demonstrates that the *size* of projection is regulated for one axon even in the absence of demonstrable qualitative specificity. Although the mechanism(s) responsible for this quantitative specificity are not presently known, the study of tonic motor axons in this and other muscles should provide a useful tool for their further investigation.

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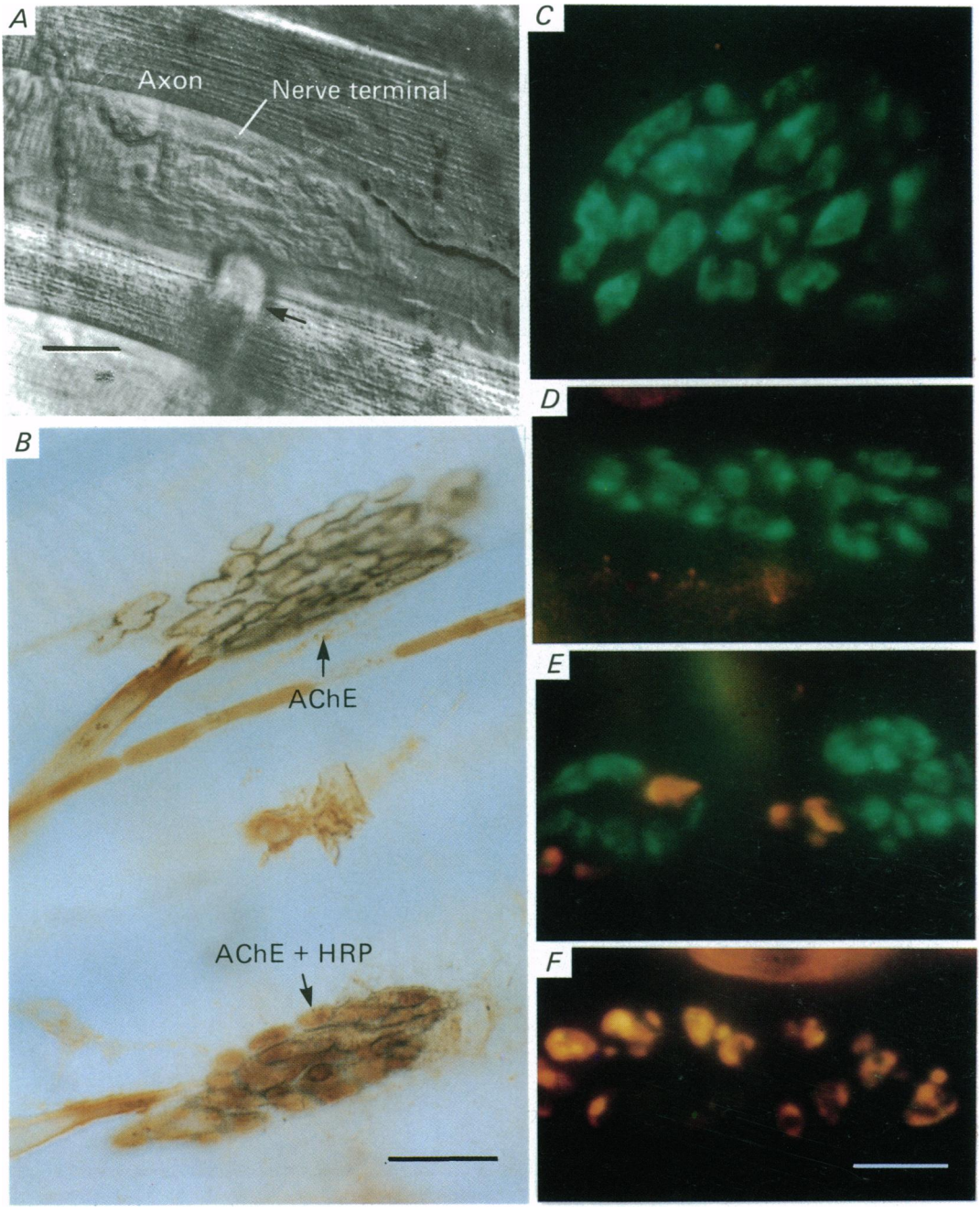
EXPLANATION OF PLATES

PLATE 1

A, extracellular stimulation pipette (arrow) positioned near a nerve terminal innervating a twitch fibre in the transversus abdominis. Stimulation of one terminal of an axon activated that axon's entire terminal arbour (Cliff & Ridge, 1973). Nomarski contrast photomicrograph; scale bar is 30 μm. *B*, two end-plates in a muscle in which a twitch axon was stimulated from an end-plate in the presence of horseradish peroxidase (HRP). The fixed preparation was stained for cholinesterase (AChE) and HRP. End-plates were located using the black AChE reaction product as a postsynaptic marker, and then scored for the presence of brown HRP reaction product in the presynaptic terminals. In the end-plate below, each AChE-stained postsynaptic specialization surrounds a terminal bouton filled with HRP reaction product, indicating that the underlying muscle fibre belonged to the stimulated motor unit. A nearby end-plate, above, is free of HRP and therefore did not belong to the stimulated motor unit. Scale bar is 20 μm. *C–F*, four classes of end-plates present in muscles where the terminal arbour of one tonic axon was studied. The muscle nerve was stimulated supramaximally with the green activity-dependent probe added to the bath; this labelled all nerve terminals in the muscle green (viewed with FITC epifluorescence optics). After rinsing, a single tonic axon was stimulated in the presence of the red probe. This labelled that axon's terminals red (using rhodamine epifluorescence optics). Shown are superimposed video micrographs taken with FITC (green) and rhodamine (red) optics, displayed on a colour monitor as red and green, respectively. Doubly labelled terminal boutons (red + green) appear yellow. Large twitch style terminals (*C*) were comprised exclusively of green boutons, indicating that the separately stimulated axon supplied no innervation to twitch fibres. Terminals innervating tonic fibres (*D–F*) were smaller and more narrow. About 50% were not innervated by the stimulated axon (*D*, all boutons green but none yellow), about 30% were partially innervated (*E*, some but not all boutons yellow), and about 20% were exclusively innervated by the axon (*F*, all boutons yellow). Scale bar, 10 μm (*C–F*).

PLATE 2

Montage of the entire terminal arbour of one twitch motor axon in the transversus abdominis, labelled by the activity-induced uptake technique. One axon was stimulated (from the terminal at far right) in the presence of the activity-dependent probe sulphorhodamine 101. This axon innervated nineteen end-plate sites (circled); each nerve terminal is also shown at higher magnification adjacent to the circled regions. Each terminal innervated a different type F (faster twitch) muscle fibre. Because the probe also non-specifically labels myelin, the branching pattern of the entire muscle nerve (except for the purely tonic end-plate bands, which are not shown) is visible. Montage of about seventy-five photomicrographs (rhodamine epifluorescence optics). Scale bar, 500 μm.


A
Axon
Nerve terminal
B
AChE
AChE + HRP
C
D
E
F