# MOTOR NEURON PROJECTION PATTERNS AND MATURATION OF MOTOR UNIT TYPES IN THE RABBIT SOLEUS MUSCLE

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## Abstract

The nervous system contains specific connections reflecting different types of projection patterns. Some of these projections have a topographic arrangement, while others have a diffuse pattern. Projections from spinal cord motor pools to different muscles are topographic. At a finer scale, patterns of projections within several muscles are topographic. These muscles tend to be flat and/or segmented. This thesis presents an investigation of the projection pattern in the innervation of the rabbit soleus muscle, which is compact and has only one tendon of origin and one tendon of insertion. Using intracellular recording of endplate potentials, tension overlap between pairs of ventral root filaments, and retrograde labeling of motor neurons following small injections of horseradish peroxidase into different regions within the muscle, it was shown that the soleus receives diffuse innervation from the spinal cord. It is thus likely that topography is related to muscle function, and that it correlates with spatial heterogeneity within muscles.

Another type of specificity in connections to muscles is that between fast and slow motor neurons and their corresponding muscle fiber types. These connections form distinct types of motor units. We have investigated the maturation of motor unit types during postnatal synapse elimination.

The ratio of motor unit tension at polyinnervated ages to that at singly innervated ages has been used to estimate the degree of polyinnervation for fast versus slow muscle fibers. Twitch and tetanic tension yield conflicting results. This contradiction was resolved using latencies to endplate potentials as an indicator of muscle fiber type. We found that fast and slow muscle fibers are polyinnervated to the same extent during both early and intermediate stages of synapse elimination, implying that specific tension, and not polyinnervation, changes differently in fast versus slow muscle fibers. These changes are consistent with those found in twitch/tetanus ratios. During synapse elimination, the twitch/tetanus ratios for fast motor units increase while those for slow motor units decrease. Furthermore, these intracellular recordings suggest a high degree of specificity at birth, which is further refined during synapse elimination.

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# CHAPTER 1:

## General Introduction

*Introduction*

Many fundamental issues in developmental biology are concerned with how sequential divisions of a single cell give rise to a diversity of cell types and their organization into different systems seen in the adult. Development of the nervous system presents especially intriguing questions because of the specificity with which neurons are interconnected. Developmental mechanisms which give rise to this specificity may be important in understanding the functional significance of the connectivity in adults. This thesis focusses on specific connections between motor neurons and muscle fibers, including those which involve topography as well as those which involve matching between fast and slow motor neurons and their corresponding muscle fiber types. Some possible mechanisms which could bring about this specificity during development will also be considered. These issues will be discussed with reference to connectivity in other systems, in which connection patterns are well characterized.

An interesting aspect of specific connections is the way that areas in the nervous system are mapped onto their targets. In one type of topographic map, the neighbor relationships of neurons in one area are preserved in the target area. This organization is found in the retinotectal and retinogeniculate projections, in the geniculostriate projection, and to some extent in higher cortical visual areas (Felleman and Van Essen, 1991), in projections to somatosensory thalamus and cortex (Penfield and Boldrey, 1937; Kaas et al., 1979), as well as in a number of other projections. In these systems, the

somatosensory thalamus and cortex (Penfield and Boldrey, 1937; Kaas et al., 1979), as well as in a number of other projections. In these systems, the spatial information presented to the sensory epithelium is preserved in higher areas. In a second type of map, exemplified by somatosensory representations in the cerebellar cortex (Shambes et al., 1978), topography is continuous within patches, although between patches there are abrupt breaks in continuity. A third type of map lacks continuous topography and contains scattered representations. One example of this type of map is the olfactory system, in which the projection from the olfactory bulb to the piriform cortex is not topographically organized (Haberly and Price, 1977). Thus, projections differ greatly in the nature and the degree of topographic organization.

These differences in mapping may be functionally significant. In sensory systems, the type of mapping from receptors to cortex may be related to the importance of spatial information processing for a given sensory modality. The visual system requires processing of detailed spatial information in order to produce a continuous representation of the visual world. In the somatosensory system, detailed spatial information is important within a given region of the body; less detailed interactions between distant parts of the body may also be important. On the other hand, the olfactory system does not process continuous features. Nelson and Bower (1990) have discussed the similarity between different neural maps and those used to optimize performance in

parallel computers; in both, the continuity of the map is related to the relative importance of local vs global interactions in a given process. Thus, the mapping seems to be optimally suited for the function of each sensory system. Given the correlation of maps with function in sensory systems, the study of projection patterns to muscles may give an indication of certain aspects of muscle function.

An interesting question regarding the mapping of specific connections is how they arise during development. In general, mechanisms which bring about specificity fall into two categories. In one class of mechanism, connections between neurons and their targets are specific from the outset. Initial specificity could arise because neurons recognize identifying molecules on target cells (Sperry, 1963). Spatial arrangements of neurons, such as selective fasciculation of axons or proximity of appropriate targets, could also be involved in establishing initial specificity (Fraser, 1989). Another possibility is that temporal differences in the development of targets distinguish them from inappropriate targets during synapse formation (see Fraser and Perkel, 1990). In the second class of mechanism, initial connections are not specific, and errors are corrected subsequent to initial synapse formation. Refinement could result from death of inappropriately projecting neurons, or from removal of inappropriate synapses (Simon and O'Leary, 1992). While adult mammalian skeletal muscle fibers receive only one input, neonatal muscle fibers are

polyinnervated; excess synapses are eliminated systematically during the first few postnatal weeks until only one synapse remains at each muscle fiber (Redfern, 1970; Brown et al., 1976). Thus, it is possible that inappropriate connections in muscle are selectively removed during postnatal synapse elimination. Alternatively, refinement could involve respecification of targets according to the activity patterns of the innervating neurons. As a background to discussing these mechanisms in muscle development, it is useful to consider other systems in which the development of specificity has been studied in detail.

*Retinotectal projections*

One pathway whose development has been particulary well studied is the retinotectal projection of lower vertebrates. In this projection, axons along the dorsoventral axis of the retina are mapped onto the lateromedial axis of the tectum, and axons along the temporonasal axis of the retina are mapped onto the rostrocaudal axis of the tectum. A number of experiments provide evidence for a role for each of several developmental mechanisms to account for this topographic ordering of connections.

Sperry (1963) proposed the existence of chemospecific markers which direct axons to appropriate synaptic sites within the target. Evidence for such a

mechanism comes from denervation experiments in which retinal axons grow past denervated tectum to find their appropriate targets, and from experiments in which retinal axons find their appropriate innervation sites on ectopically grafted portions of tectum (Yoon, 1972). These chemical markers do not need to specify individual connections; two orthogonal gradients of markers may suffice. In support of this view, Trisler and coworkers (1981) have identified TOP molecules, which are expressed in gradients in the retina and tectum.

While chemospecificity is likely to be involved in establishing this topography, interactions between fasciculating axons may also be involved to some extent. Disruption of fascicles using antibodies that bind to the neural cell adhesion molecule (NCAM) results in a disordered map (Thanos et al., 1984). Errors are partially corrected, presumably in response to cues present on the tectum. The authors therefore suggest that both types of mechanisms are involved. Moreover, evidence for selective fasciculation between neighboring axons comes from tissue culture studies, in which growth cones from temporal chick retina explants preferentially grow along temporal axons and along anterior tectal membranes; however, growth cones from nasal retina explants grow equally well along nasal and temporal explants (Bonhoeffer and Huf, 1980; Walter et al., 1990). The selectivity in temporal axon growth may be guided by an avoidance mechanism; when posterior tectal membranes are inactivated by heat or enzymes they no longer repel growing temporal axons

(Walter et al., 1990). These results suggest a role for selective fasciculation, but at the same time demonstrate that other mechanisms are likely to be involved.

The establishment of topographic ordering may require plasticity within the retinotectal projection. When half the retina is removed, retinal axon terminations expand onto the whole tectum (Schmidt et al., 1978); when a retina with two nasal regions is constructed, the entire tectum is innervated in a topographic fashion (Feldman and Gaze, 1975). Thus, matching between parts of the retina and parts of the tectum is not unalterable. Plasticity is not merely an artifact of experimental perturbations, however, as it occurs even in normal development. For example, retinal terminations remodel during development to accomodate for the fact that the retina grows concentrically while the tectum grows along the caudomedial border (Gaze and Sharma, 1970; Reh and Constantine-Paton, 1984). Refinements of the topographic projection following a more diffuse initial projection may also depend on neuronal activity (Schmidt and Edwards, 1983; Hartlieb and Stuermer, 1989) and may use information about correlated activity between retinal ganglion cells (Schmidt and Eisele, 1985; see Shatz, 1990).

Several mechanisms are thus involved in establishing the topography seen in the retinotectal projection. Experimental manipulations reveal the importance of different mechanisms in different contexts. The relative importance of each mechanism has been estimated by computer simulations

which are able to simulate a wide variety of the experimental observations (Fraser and Hunt, 1980; Fraser and Perkel, 1990). These types of simulations thus provide a useful tool for understanding the mechanisms which operate in normal development to bring about the observed topographic specificity.

*Topography of projections to muscles*

Spinal cord motor neurons project to some muscles in a topographic fashion. Intercostal muscles, which lie in between the ribs, are generally innervated by ventral roots from corresponding body segments (Carpenter and Sutin, 1983). In principle, such a topographic arrangement might arise because ventral root axons simply grow to the nearest target muscle. However, Wigston and Sanes (1985) showed that growing axons have a preference for their muscles from appropriate segments, even when they are experimentally transposed to ectopic locations. In their study, intercostal muscles from several positions were transplanted to the neck region; the superior cervical ganglion was removed, and muscles were reinnervated by sympathetic preganglionic axons. Although sympathetic axons do not normally innervate skeletal muscles, they made functional connections during reinnervation, and muscles were selectively reinnervated by axons from spinal segments corresponding to the original location of the muscles. These results suggest that intercostal

muscles express markers indicating their rostrocaudal position. Consistent with this view, others have found antibodies that bind to the nerves innervating intercostal muscles in a rostrocaudal gradient (Suzue et al., 1990).

Topography in muscle projections is not limited to segmental muscles of the trunk. In the chick hindlimb, there is a correlation between the mediolateral position of motor pools (the collections of motor neurons innervating single muscles) within in the spinal cord and the dorsoventral position of the innervated muscles within the limb (Landmesser, 1978). These projections seem to be specific at the outset; specificity is evident even before the embryonic muscle masses have cleaved to form individual muscles. Moreover, this specificity is evident before the period of programmed cell death of motor neurons, and thus does not require selective death of inappropriately projecting motor neurons. The role of chemospecific recognition has been examined using anterograde tracing studies with horseradish peroxidase (HRP), which have shown that axons enter the correct plexus and major nerve trunks with few errors (Lance-Jones and Landmesser, 1981a; cf. Lamb 1979). Axons are randomly organized in spinal nerves, but begin to form clusters as they pass through the plexus (Tosney and Landmesser, 1985). The persistence of specificity in arbor patterns following embryonic spinal cord reversal or displacement (Lance-Jones and Landmesser, 1980, 1981b) suggests that molecular cues are involved in specifiying axonal pathways through the plexus.

While these examples illustrate topography in projections to groups of muscles, other evidence suggests that at least some projections within muscles are also topographic. Examples include the diaphragm and the serratus anterior muscles, which are divided into rostrocaudal sectors within each muscle (topography of projections within individual muscles is discussed in more detail in Chapter 2). Like the intercostal muscles, these muscle sectors insert into ribs and also receive innervation from corresponding spinal segments (Laskowski and Sanes, 1987). The topographic projections to the serratus anterior and diaphragm muscles are evident at birth, although the pattern is not as precise as it is in adults. In these topographically innervated muscles, as well as some others, topography is more pronounced following the period of postnatal synapse elimination (Brown and Booth, 1983; Bennet and Ho, 1988; Laskowski and High, 1989). Thus, selective synapse elimination may be involved in refining topography.

*Specific innervation of motor unit types*

Another type of specific connectivity is that seen between spinal cord motor neurons and particular types of muscle fibers within a muscle. The functional unit of muscle is the motor unit, which consists of a motor neuron and all of the muscle fibers innervated by it. Most adult mammalian skeletal muscles are composed of a mixture of different types of motor units, which

have been grouped into two main types, fast and slow, based on differences in histochemical and physiological properties. There is considerable evidence that histochemical and physiological properties of motor units are correlated, and, furthermore, that the muscle fibers innervated by a single axon are all of the same histochemical type (Edström and Kugelberg, 1968; Burke et al., 1973). Thus, fast and slow motor units represent two distinct classes of functional units which are specialized for different kinds of activation within a muscle. In order to understand the functional significance of fast and slow motor units and their specific connectivity, it is necessary to examine their underlying histochemical and physiological characteristics in more detail.

*Histological differences between motor unit types*

Fast and slow muscle fibers contain different isoforms of myofibrillar ATPase, one of the proteins involved in contractile mechanisms. Histochemical reactions for this protein can distinguish muscle fiber types anatomically (Guth and Samaha, 1969). In addition, immunohistochemistry using monoclonal antibodies against type-specific myosin forms (Gauthier and Lowey, 1979) and troponin forms (Dhoot et al., 1978; Dhoot and Perry, 1980) have also revealed differences between fiber types. Muscle fiber types can also be distinguished using histochemistry which reveals differences in glycogen content and in the activity of a variety of metabolic enzymes including malate dehydrogenase,

lactate dehydrogenase, and succinic dehydrogenase (Peter et al., 1972; for review see Pette and Staron, 1988). These histochemical diffences between fiber types are significant in that they may directly reflect biochemical mechanisms underlying some of the physiological differences between motor units.

In some cases, histochemical staining is correlated with muscle fiber size, or cross-sectional area; however, this difference is not consistent across muscles. In general, slow muscle fibers have a smaller diameter than fast muscle fibers; the correspondingly larger surface area to volume ratio may be related to an increase in access to oxygen from the blood supply (Burke, 1981). However, there are many exceptions to this trend. In the rabbit soleus, for example, slow muscle fibers have a larger cross-sectional area than fast muscle fibers (Gordon, 1983). Thus, while differences in size can be significant between fiber types, these differences do not consistently correlate with function.

*Physiological differences between motor unit types*

Differences between fast and slow motor units based on muscle fiber histochemistry and anatomy are accompanied by differences in a variety of physiological characteristics, which reflect functional aspects of motor unit types. One such difference is in the motor unit twitch contraction times, i.e., the

rise time to peak. In a given muscle, fast motor units have shorter contraction times than slow motor units, although the range of contraction times varies across muscles. Contraction times for motor units correlate with ATPase staining (Barany, 1967; Kugelberg, 1973); this correlation is consistent with the hypothesis that myofibrillar ATPase activity is a determinant of muscle fiber shortening speed.

In addition to differences in contraction speed, motor units differ in their fatiguability. In fast motor units, tension in response to continued tetanic stimulation of ventral root axons declines more rapidly than in slow motor units. In general, faster contracting muscle fibers are less resistant to fatigue, and seem to be specialized for high intensity contraction of short duration. Differences in fatiguability have been shown to correlate with high and low ATPase acitvity, respectively (Kugelberg, 1973). Although resistance to fatigue is correlated with succinic dehydrogenase activity in some cases (Edström and Kugelberg, 1968), in the rat soleus higher activity is correlated with fast motor units (Kugelberg, 1973); thus other factors are probably also involved in determining fatiguability. In addition, some motor units with fast contraction times are somewhat fatigue-resistant, forming the basis for a subdivision of the fast motor unit class into fast fatiguable (FF) and fast fatigue-resistant (FR) types (Burke et al., 1973).

While slow motor units are more resistant to fatigue, they tend to generate less force than fast motor units. Both twitch and tetanic tension have smaller amplitudes in slow motor units than in fast motor units. The amplitude of motor unit tension is the product of the number of muscle fibers in the unit, the cross-sectional area of the muscle fiber, and the specific tension (tension per unit area) of muscle fibers. Some studies have shown that specific tension is about twofold higher in fast muscle fibers than in slow muscle fibers (Burke and Tsairis, 1973; Close, 1972). However, as noted above, relative diameters of muscle fiber types are not consistent across muscles. Thus, the relative contributions of these factors in producing differences in motor unit tension are not clear for all muscles.

Differences in functional characteristics of motor unit types can be further described by the twitch/tetanus ratio, which is independent of the number of fibers in a motor unit. This ratio indicates how much of the maximum tetanic motor unit tension is attainable in response to a single stimulus. The twitch/tetanus ratio is higher for fast motor units than slow motor units in the cat medial gastrocnemius (Burke et al., 1973). However, Bagust (1974) reports that the reverse trend is seen in adult cat and rabbit soleus; i.e., twitch/tetanus ratios increase with twitch rise times in these muscles.

While these physiological differences between motor units are generally the result of properties of the muscle fibers contained in each motor unit, there

are also differences in the innervating motor neurons. Fast motor neurons generally have a larger cell body size than slow motor neurons (Cullheim and Kellerth, 1976); however, the distributions overlap substantially. Axonal conduction velocity, which is related to cell body size (Cullheim, 1978), is also somewhat faster in fast motor axons than in slow motor axons (Bagust et al., 1973; Bagust, 1974; Wuerker et al., 1965). Moreover, fast and slow motor neurons differ in their firing patterns. Fast motor neurons have phasic firing patterns; i.e., they in short bursts of high frequency, while slow motor neurons fire tonically, in longer bursts of lower frequency (Denny-Brown, 1929; Hennig and Lømo, 1985).

Within a motor pool, there is a correlation between the recruitment threshold (in response to primary afferent stimulation) and the size (tension) of motor units (Henneman, 1957). Consistent with the correlation between tension and motor unit type, recruitment thresholds are lowest for slow motor units and highest for fast motor units; however, recruitment ordering rules may be different when secondary afferents are also considered (for review, see Burke, 1981). That fast and slow motor units have different recruitment thresholds provides a mechanism by which fast and slow muscle fibers can be differentially activated within a single muscle. This differential control is important because individual muscles participate in different types of movement. In general, fast motor units are involved in strong contractions of

short duration, such as those required in locomotion, while slow motor units participate in less demanding contractions of long duration, such as those required in postural control (see Burke, 1981). Moreover, the correlation of recruitment thresholds with motor unit size, which exists even within fast and slow subpopulations, allows further control over the extent of activation, and thus provides control over a broad dynamic range within a single muscle.

*Development of fast and slow muscle fibers*

In order to understand the development of these motor unit classes, it is first useful to consider the early development of muscle fiber types. The earliest precursors of skeletal muscle are the myoblasts, which fuse to form multinucleate myotubes. At this stage, muscles have not differentiated and are present as muscle masses in the limb. A study of chick myogenesis (Noakes et al., 1986) showed that the main nerve trunk enters the muscle masses before myotubes are formed; subsequently, the myotubes form clusters which will form distinct muscles. Myotubes form at two distinct times in development (Ontell and Kozeka, 1984; Ross et al., 1987a). In the rat, primary myotubes fuse at embryonic day 17. Myoblasts form in clusters around these primary myotubes, and begin to fuse at embryonic day 19, forming secondary myotubes. The first synapses are made after the primary myotubes are formed but before the secondary myotubes are formed (Bennett and Pettigrew, 1974).

While fused myotubes express myosin, the isoforms of myosin differ in embryonic, fetal, and adult muscle (Hoh and Yeoh, 1979; Whalen et al., 1981; Butler-Browne and Whalen, 1984), and the activity of myofibrillar ATPase changes between birth and maturity (Pelloni-Mueller et al., 1976). Differences between fast and slow myosin isoforms are evident in fetal muscle (Lyons et al., 1983), although individual myotubes may express both fast and slow forms early in development (Gauthier et al., 1978). Studies of cultured chick myoblasts ( Miller et al., 1985; Schafer et al., 1987; Stockdale and Miller, 1987) have shown that the expression of myosin isoforms is related to cell lineages in myoblast production. Cultures prepared from early myoblasts form myotubes which express fast, slow, or fast and slow myosin; cultured myoblasts from older animals form myotubes which express only fast myosin. Although myotubes may transiently express several forms of myosin, eventually the primary myotubes become slow muscle fibers and secondary myotubes become fast muscle fibers (Rubinstein and Kelly, 1981).

The development of myotubes and the subsequent changes in myosin expression may be dependent on innervation. Ross et al. (1987a) suggested that the occurrence of neuronal cell death during the interval between primary and secondary myotube formation reflects a regulatory role of neurons on the number of secondary myotubes. Experiments on aneurogenic chicks (Butler et al., 1982) and bungarotoxin-treated rats (Ross et al., 1987b) have shown that

innervation and nerve transmission are required for formation of secondary myotubes, possibly because mitosis and fusion in the later population of myoblasts requires innervation and/or contraction of primary myotubes. While secondary, but not primary, myotube formation is nerve-dependent, both fast and slow muscle fibers develop from primary myotubes in the absence of functional innervation (Butler et al., 1982; Butler-Browne et al., 1982; Phillips et al., 1986; cf. Rubinstein and Kelly, 1978). Mouse myotubes cultured without nerves undergo changes in myosin expression at a time scale comparable to that seen in vivo (Silberstein et al., 1986). The nerve-independent formation of both fiber types from primary myotubes is consistent with the finding that early myoblasts can produce both fast and slow myosin (Miller et al., 1985; Schafer et al., 1987; Stockdale and Miller, 1987); however, it is apparently in contradiction with the observation that primary myotubes later become slow muscle fibers (Rubinstein and Kelly, 1981). While the formation of primary myotubes is nerve-independent, primary myotubes may require innervation later in development to restrict their myosin expression to the slow isoform. Thus it is likely that the role of innervation in the development of muscle fibers varies temporally as well as according to fiber type.

Evidence that neuronal activity can influence muscle fiber type comes from cross-reinnervation studies, in which nerves from predominantly fast and slow muscles are forced to reinnervate muscles of the opposite type, and from

experiments in which fast and slow muscles were chronically stimulated at low and high frequencies, respectively (Bagust et al., 1974b; Salmons and Sreter, 1976; Jolezc and Sreter, 1981; Gorza et al., 1988). In these experiments, the fiber type composition (revealed by ATPase assays) and the mean twitch contraction time were transformed to correspond with the reinnervating nerve type.

Although these experimental manipulations are non-physiological, the ability of motor neurons to specify fiber type has also been implicated during normal development. For example, such a mechanism may be involved in changes in fiber type composition subsequent to the period of postnatal synapse elimination. The soleus muscle in the rat (Kugelberg, 1976) and rabbit (Gordon, 1983) contains at least as many fast muscle fibers as slow muscle fibers in the neonate; however, after 5 postnatal weeks the fiber type composition begins to change and by 34 weeks, most of the muscle fibers are slow. Two possible mechanisms have been proposed to account for this transformation (Kugelberg, 1976; Gordon, 1983). In one, entire motor units change their type in response to changes in the firing pattern of the innervating motor neuron; in the other, slow motor axons sprout and innervate fast muscle fibers, displacing fast motor inputs and changing the muscle fibers from fast to slow. While it is not known to what extent these mechanisms are actually used, both rely on neuronal respecification of muscle fiber types. Thus, it is likely that

the influence of motor neurons on fiber type is relevant to normal development.

*Specificity of connections*

Differential control over muscle fiber activation relies on the fact that fast and slow muscle fibers are separated into different motor units. The purity of motor units (Edström and Kugelberg, 1968) implies that, in adults, fast and slow motor neurons have synaptic connections predominantly with the corresponding muscle fiber types. This section will examine some possible developmental mechanisms which bring about this specificity of connections.

A number of possible developmental mechanisms for specificity between motor neurons and muscle fibers have been proposed; these fall into two broad classes. At one extreme, connections may be completely specific from the outset; at the other, initial innervation may be random and specificity may be refined by neuronal specification of fiber types or by selective removal of inappropriate synapses. It is unlikely that either type of mechanism by itself accounts entirely for specificity.

Several studies have provided evidence that neonatal soleus motor units are biased toward one type of muscle fiber, although this specificity is not as precise as in the adult. Neonatal rabbit soleus motor units have a bimodal distribution of twitch rise times (Gordon and Van Essen, 1985), suggesting that motor units contain predominantly one type of muscle fiber; random innervation

would have resulted in a unimodal distribution. Similarly, considerable specificity has been demonstrated in 8 and 16 day old rats. Glycogen depletion of motor units along with monoclonal antibodies specific to myosin isoforms to identify fiber types have shown that individual motor units are dominated by fast or slow muscle fibers (Thompson et al., 1984; cf. Jones et al., 1987). A similar finding was reported in the 2 day mouse soleus (Fladby and Jansen, 1990) in which muscle fibers belonging to a motor unit were identified with recordings of endplate potentials and filled with an anatomical tracer. All of these findings are consistent with a bias for appropriate muscle fiber types at an early age.

The presence of neonatal specificity suggests that initial innervation, which occurs prenatally, may not be random. Because specificity is evident at an age when muscle fibers are polyinnervated, it is unlikely that neuronal activity alone accounts for segregation of fiber types; individual fibers would receive inputs from neurons with different activity. Moreover, the firing patterns of motor neurons in neonates are not as differentiated as they are in adults (Navarrete and Vrbova, 1983). The extent to which synapse elimination refines specificity is unclear. While Thompson et al. (1984) did not find any improvement in the homogeneity of rat motor unit types following synapse elimination, Fladby and Jansen (1990) found that motor units were more homogeneous in 2-week old mice, which had undergone synapse elimination, than in neonates, and that appropriate synapses were at a competitive

advantage during the elimination of synapses. That these two studies disagreed on the role of synapse elimination in refining specificity may be related to the fact that neonatal mice are developmentally less mature than neonatal rats. Furthermore, the homogeneity at birth was higher in rats (>70%) than in mice (>50%); improvement in mice might have been easier to detect. Thus, a parsimonious view is that the initial formation of connections is considerably specific, and that synapse elimination further refines this specificity.

As with other systems already discussed, specificity of neuronal connections may be directed by chemospecific matching between fast and slow motor neurons and their respective targets. The possibility that fast and slow motor axons recognize muscle fiber types via chemospecific markers has been examined during reinnervation. In adults, reinnervating motor axons initially make synapses with muscle fibers randomly; later, motor unit homogeneity is restored by neural respecification of muscle fibers (Kugelberg, 1976). This lack of recognition does not necessarily contradict the existence of chemospecific markers in development because the expression of these molecules may decline with age. In a study of neonatal rat soleus, motor axons preferentially reinnervated one type of muscle fiber almost as selectively as those in normal muscles (Soileau et al., 1987), supporting the view that muscle fibers are chemically marked. However, in a similar study of neonatal rabbit (Soha et al.,

1989), reinnervation was not specific and resulted in a unimodal distribution of twitch rise times. The apparent contradiction between these two results may have arisen because rather different methods were used; alternatively, it may reflect differences in the maturational state of rabbits and rats at birth such that rabbits may have lost their chemospecific markers by this time. Thus, while results from these studies conflict, there may be evidence for chemospecific recognition between motor neurons and muscle fibers.

Another possibility is that specificity arises because of temporal differences in synapse formation between fast and slow motor units. Because the myotubes which become fast and slow muscle fibers develop at two distinct times, a corresponding lag in axon outgrowth between fast and slow motor neurons could result in specific connections (Rubinstein and Kelly, 1981). The first axons arrive when only the primary myotubes have formed (Bennett and Pettigrew, 1974), and synapses at primary myotubes mature before those at secondary myotubes (Rubinstein and Kelly, 1981). However, it is not known whether the early and late synapses correspond with slow and fast axons.

Specificity of connections between motor neurons and appropriate muscle fiber types may involve a combination of all of the above mechanisms, with different mechanisms acting to different extents at different times during development. While muscle fiber types are expressed in the absence of innervation in experimental situations, the activity of innervating neurons may

have a role for refining specificity later in normal development, and synapse elimination may participate in refining the homogeneity of muscle fibers within a motor unit. Because various studies have shown the importance of some of these mechanisms in certain experimental contexts, and not in others, it is essential to consider how different mechanisms may work in combination to give rise to the adult pattern of specificity. Further study, perhaps including simulations of combined mechanisms, will be necessary before the development of this system is understood.

**Summary of thesis chapters**

Mapping of connections from the spinal cord, and development of specific motor unit types have been explored in the immature rabbit soleus muscle. Chapter 2 examines the pattern of projections to the rabbit soleus muscle. This study was motivated by evidence for topography in the projections within some individual muscles, and by evidence that the soleus motor units in extreme roots lose more synapses during synapse elimination than do motor units in middle roots (Callaway et al., 1989). While other muscles that have been shown to receive topographic projections share some distinct anatomical features, such as a sheetlike arrangement or segmentation, the soleus muscle lacks these features. Thus it is of interest to know whether

topography is a general feature of muscle innervation, or if it is reserved for muscles with these anatomical features. Additionally, the role of selective synapse elimination in refining topography was examined in the developing soleus muscle.

Intracellular recording of muscle fibers following stimulation of different ventral root filaments, tension overlap between adjacent and distant ventral root filaments during polyinnervation, and retrograde labeling of spinal cord motor neurons following local injections of tracer into the muscle suggest that the pattern of the projection to the soleus muscle is diffuse. The variability in the types of maps to muscle suggests that continuous topographic muscle projections are reserved for muscles with particular anatomical features, and furthermore, that synapse elimination does not produce topography unless it is present, at least crudely, in the initial projection.

Despite the lack of topography in the soleus, specific connections are present between fast and slow motor neurons and their corresponding muscle fiber types. In Chapter 3, the development of motor units is further examined. This study shows that twitch/tetanus ratios are higher for slow motor units than for fast motor units in polyinnervated muscles, and that these ratios subsequently change differently in the two motor unit types so that after synapse elimination, the ratios are higher for fast motor units than for slow motor units. This finding provides further evidence that some properties of fast

and slow motor units do not emerge until later in postnatal development.

While twitch/tetanus ratios are not mature at birth, experiments described in Chapter 3 show that fast and slow motor axons differ in their conduction velocities. These differences were used to identify muscle fiber types during intracellular recording. The number of inputs per muscle fiber were counted, and it was found that, contrary to predictions from relative declines in twitch tension or in tetanic tension, fast and slow muscle fibers are polyinnervated to the same extent at the earliest ages examined. A plausible explanation for the conflicting estimates is that specific tension (tension per unit area) changes differently for fast versus slow motor units, and that these changes are different for twitch versus tetanic specific tensions. This view is consistent with the results on twitch/tetanus ratios, and provides further insight into the nature of maturation of muscle fiber and motor unit types.

# CHAPTER 2:

## Lack of topography in the spinal cord projection to the rabbit soleus muscle

## Introduction

The pattern of projections from the spinal cord to the body musculature is grossly topographic, in that the positions of motor pools are related to the positions of muscles in the body or in a given limb (Sherrington,1892; Browne, 1950; Landmesser, 1978). For at least some muscles there is topography at a finer scale, in that the positions of motor neurons within the motor pool are related to the distribution of target muscle fibers within the muscle. Topographically innervated muscles tend to have characteristic anatomical features. Some are flat, sheet-like muscles such as the diaphragm (Laskowsksi and Sanes, 1987) and the gluteus (Brown and Booth, 1983). Some are segmented muscles, such as the diaphragm and serratus anterior, which insert into several ribs along body segments. The rostrocaudal position of the segments is related to the rostrocaudal position of neurons innervating each segment (Laskowski and Sanes, 1987). Because different segments may be activated differently during normal behavior, the observed topography may reflect functional partitioning of the muscle.

In the experiments reported here, we tested for topographic organization of the soleus muscle, which differs in several respects from muscles which have been shown to be topographic. It is thick, rather than sheet-like, and has one tendon of origin and one of insertion; thus it is not segmented. Although

the soleus lacks the anatomical features generally found in muscles that are topographically innervated, we had reasons to suspect that the rostrocaudal positions of soleus motor neurons might be related to the positions of their target muscle fibers. First, over the course of synapse elimination, the extreme soleus motor units (those from the rostral and caudal extremes of the motor pool) lose significantly more synapses than middle motor units (Callaway et al., 1989). Thus, there is some segmental specificity involved in the development of projections to the soleus. Secondly, it has been shown that the soleus muscle develops a spatial gradient in the ratio of fast and slow muscle fibers (Gordon, 1983). In the frog gluteus muscle, such a gradient in the muscle is related to rostrocaudal bias in the innervation of the muscle (Brown and Everett, 1990).

A necessary condition for topography to occur in muscle innervation is that the muscle fibers belonging to each motor unit be restricted to part of the muscle, rather than dispersed throughout it. The extent of muscle occupied by each motor unit is not known for the rabbit soleus; however, glycogen depletion studies in the comparably sized cat soleus show that motor units are restricted to about 50% of the total cross sectional area of the muscle, with a range from 41 to 76% (Bodine et al., 1988). Soleus motor units are thus only modestly restricted compared to motor units of other muscles, such as the tibialis anterior in cats, in which motor unit territories range from 8 to 22% of the muscle

(Bodine et al., 1988).

Changes in topography have been studied during postnatal development. In mammalian skeletal muscle, each muscle fiber is polyinnervated at birth, and excess inputs are systematically removed until only one input remains on each fiber by about two postnatal weeks (Brown et al., 1976). In the rabbit soleus, each motor neuron innervates about 5% of the muscle fibers at birth; this figure reduces to about 1% following postnatal synapse elimination. This reduction in motor unit size can potentially establish or refine topography in a projection to a muscle. In the cat lateral gastrocnemius (Bennett and Ho, 1988), and in the rat serratus anterior and diaphragm (Laskowski and High, 1989) topography is more pronounced following the period of synapse elimination. In our study of topography in the soleus muscle, we also sought to determine whether or not synapse elimination plays a role in establishing or refining topography.

Three different approaches were used to assess the topography of the soleus innervation. In the first approach, intracellular recordings of endplate potentials in early and intermediate age rabbits were used to assess the spinal origin of inputs to two distinct regions of the muscle. Neither region was biased in innervation toward rostral or caudal portions of the motor pool. These experiments also showed that individual muscle fibers frequently receive separate inputs arising from widely separated regions of the motor pool. In

another set of experiments, physiological measurements of tension overlap in young, polyinnervated muscles were used to test whether neighboring motor neurons in the spinal cord are more likely than distant ones to have shared innervation of target muscle fibers. Our findings suggest that the relative positions of motor neurons in the spinal cord do not correlate with the extent to which neurons share muscle fibers. The third set of experiments, performed at both early and late stages of maturation, used retrograde labeling of motor neurons following local injections of tracer into muscle to ask whether local regions of muscle are innervated by motor neurons that are clustered in the spinal cord, and whether the positions of local injections into muscle are correlated with the positions of labeled regions within the motor pool. Clustering was not apparent: small and large local injections resulted in comparably dispersed staining of motor neurons within the motor pool. Moreover, the rostrocaudal position of labeled neurons was not correlated with the position of the injection site within the muscle. Together, these results provide evidence that the soleus muscle is not topographically innervated. Furthermore, synapse elimination in this muscle does not improve topography. Thus, the soleus muscle is unlike flat, segmented muscles, in that partitioning of function in the soleus is not likely to be related to the rostrocaudal position of soleus motor neurons.

**Methods**

Pregnant New Zealand White rabbits were obtained from ABC Rabbitry (Pomona, CA). Litters were housed with their mothers. Two age groups were used in this study. The early age group, 4-5 days postnatal, have completely polyinnervated soleus muscles, while the late age group, 13-16 days, have soleus muscles that are singly innervated at most or all muscle fibers (Gordon and Van Essen, 1985).

The soleus muscle was isolated, and its nerve supply was dissected back to the spinal roots in 15$^{o}$C Ringer's solution (150mM Na, 5mM K, 1mM Mg, 5mM Ca, 167mM Cl, 16mM glucose, and 14mM HEPES buffer, at pH 7.4) perfused with oxygen. The rabbit soleus receives innervation from ventral root S1, and frequently from ventral roots L7 and/or S2 as well. All three ventral roots were included in the preparation. The ventral roots were cut at the spinal cord, and dorsal roots were cut proximal to the dorsal root ganglia. Nerve branches between roots, such as one frequently found from S1 to S2, were left intact. Ventral roots were identified by their size and position, and were cut obliquely such that the rostral part was always longer. The preparation was transferred to a dish containing Ringer's solution and was continuously aerated with oxygen.

*Tension recording*

Once in the dish, the roots had a natural splay which facilitated division of roots into filaments. The muscle was pinned to the dish at the proximal tendon. The distal tendon was tied with 6-0 silk thread to a tension gauge mounted on a micromanipulator. The tension gauge was attached to a tension transducer whose signal was amplified, displayed on an oscilloscope, and digitized and stored on an IBM PC for later analysis. The maximum twitch tension for the whole muscle was measured by stimulating along the sides of the muscle with a bipolar electrode, which was placed at an optimal position along the muscle. The length of the muscle was adjusted so as to yield the maximum twitch tension. The direct tension measurement was repeated at intervals throughout each experiment, and the experiment was discontinued if the maximum twitch tension fell below 80% of its original value.

Ventral roots were stimulated using fine-tipped polyethylene suction electrodes and the tension elicited by stimulation of each was noted. Ventral roots were divided into 5 or 6 filaments; usually 4 or more of these filaments were from ventral root S1. The rostrocaudal position of each of the filaments was recorded. Each filament was stimulated and its tension as a percentage of the maximum was recorded. We attempted to make filaments that elicited approximately equal tensions, although in order to keep the rostrocaudal position of the filaments unambiguous it was necessary to avoid excessive

division of the filaments.

*Intracellular recording*

Both tendons were removed from the muscle to reduce muscle contractions during intracellular recording. The cut muscle was pinned as flat as possible and illuminated from below. In this configuration the muscle nerve was visible for a few millimeters into the muscle. Two recording sites were used: one dorsal and one ventral to the muscle nerve. Muscle fibers were impaled with a glass microelectrode (5 to 30 MΩ) filled with 3 M KCl. Each suction electrode was stimulated in turn, and for each electrode that contributed to the endplate potential (e.p.p.) the stimulus intensity was varied to reveal any multiple components of the e.p.p. The number of inputs to each muscle fiber from each filament was recorded.

We used an index similar to that of Laskowski and Sanes (1987) to describe the average rostrocaudal position of motor neurons innervating each of the two regions within the muscle. Because the filaments used in this study were divided differently in each preparation, we expressed the rostrocaudal position of each filament as a percentile (P) of the rostrocaudal extent of the motor pool (Equation 1):

$$(1) \qquad P_k = 100 \; X \; \frac{\sum_{i=1}^{k} T_i}{\sum_{i=1}^{n} T_i}$$

where $P_k$ signifies the percentile of the kth filament of n total filaments, $T_i$ indicates the twitch tension of the ith filament as a percent of the maximum twitch tension for the muscle, and increasing values of i indicate successively more caudal filaments. These percentiles thus indicate the rostrocaudal position of the filaments: the rostral extreme is 0% and the caudal extreme is 100%. If the projection to the soleus were topographic, there would be a difference in the mean position of the inputs to dorsal versus ventral regions of the muscle. To obtain the index indicating the average rostrocaudal position of motor neurons innervating each region, we used an average of the percentiles weighted by the number of inputs to the region from each percentile (Equation 2):

$$\text{(2)} \qquad \text{Index} = \frac{\sum_{i=1}^{n} s_i P_i}{\sum_{i=1}^{n} s_i}$$

where $s_i$ indicates the number of inputs from the ith filament in a given region of the muscle. Thus an index of 50% indicates that the average rostrocaudal position of inputs to the region is halfway between the extremes of the motor pool.

*Tension overlap.*

In order to examine the pattern of projections in the young (4-5 day) rabbit soleus muscle, we took advantage of the fact that these muscles are polyinnervated. At this age, motor units can share muscle fibers, which results in a tension overlap, illustrated schematically in Figure 1. A muscle fiber that receives suprathreshold input from two motor neurons produces the same tension when one or both of the motor neurons are stimulated. Thus, when one

Figure 1. Schematic diagram illustrating how polyinnervation of muscle can lead to tension overlap of motor units. Neurons A and B share the middle muscle fiber. Tension generated by stimulation of A and B individually ($T_A$ and $T_B$) are shown in the first two traces on the right. In the third trace, the dashed line shows the arithmetic sum of $T_A + T_B$. The solid line shows the tension generated when A and B are stimulated together ($T_{AB}$). The extent to which these tensions are nonlinear is related to the extent to which motor units share muscle fibers, and can be quantified by the equation given.

$$\%Overlap_{AB} \equiv 200\left(1 - \frac{T_{AB}}{T_A + T_B}\right)$$

Figure 1

stimulates two motor units that share muscle fibers, the tension generated by this stimulation is less than the sum of tensions generated when each unit is stimulated alone. In order to use tension overlap to estimate shared innervation between filaments, a useful quantity is $T_{(A \cap B)}$, the tension produced by fibers shared by filaments A and B. Overlap can then be expressed as$T_{(A \cap B)}$ as a percentage of the mean tension in filaments A and B, or

(3) $\%\text{overlap} = 200 \times T_{(A \cap B)} / (T_A + T_B)$

where $T_A$ and $T_B$ are the tensions generated by stimulation of individual units A and B, respectively. However, because it is difficult to measure $T_{(A \cap B)}$ directly, we can use the relationship

(4) $T_{(A \cap B)} = T_A + T_B - T_{(A \cup B)}$

where $T_{(A \cup B)}$ represents the tension generated when filaments A and B are stimulated simultaneously, expressed as $T_{AB}$. Combining equations (3) and (4), we obtain an expression for tension overlap based on three experimentally

measurable parameters:

(5) $\%overlap_{AB} = 200 ( 1 - T_{AB} / ( T_A + T_B ) )$.

Percent overlap expresses the number of muscle fibers that receive inputs from both units as a percentage of the average number of fibers in each unit. Tension overlap is dependent on the presence of polyinnervation and the size of the motor units. An estimate of overlap values can be obtained as follows. Assuming a linear relationship between tension and the number of fibers contracting,

Assumption 1: $T_i = km_i$, where $m_i$ = the number of muscle fibers in filament i

an appropriate expression for % overlap is:

(6) $\%overlap_{AB,est} = 200 \text{ X } m_{(A \cap B)} / ( m_A + m_B )$

where $m_{(A \cap B)}$ = the number of muscle fibers innervated by both A and B.

From probability, we have:

(7) $m_{(A \cap B)} = Mp(AB)$

where M represents the total number of fibers in the muscle and p(AB) represents the probability that a given fiber has an input from both A and B. If we assume that all fibers are polyinnervated, and there is no specificity or topography, then p(A) and p(B) are independent and

Assumption 2: $p(AB) = p(A)p(B)$

Combining equations (6) and (7) with assumption (2), we obtain:

$$\% \text{ overlap}_{AB,est} = 200 \text{ X } m_A \, m_B / M ( m_A + m_B ).$$

For example, given that M is about 15,000 in the rabbit (E. Fung, unpublished observations), the expected overlap between two filaments that contribute 5% and 10% of the tension, respectively, would be 6.7%. Because tension overlap is proportional to the size of the unit, we used filaments that contained several clustered motor axons in order to obtain larger, more sensitive measurements.

Figure 2. Experimental set-up which used tension overlap to investigate topography. The 4-5 day rabbit soleus muscle was dissected together with its innervation back to the spinal roots, L7, S1, and S2. The muscle was pinned out and attached to a tension gauge. The ventral roots were divided into filaments which contained several soleus axons and which contributed about 20% of the total muscle twitch tension. The rostrocaudal position of the filaments was noted. Percent overlap between adjacent filaments was compared to that between distant filaments.

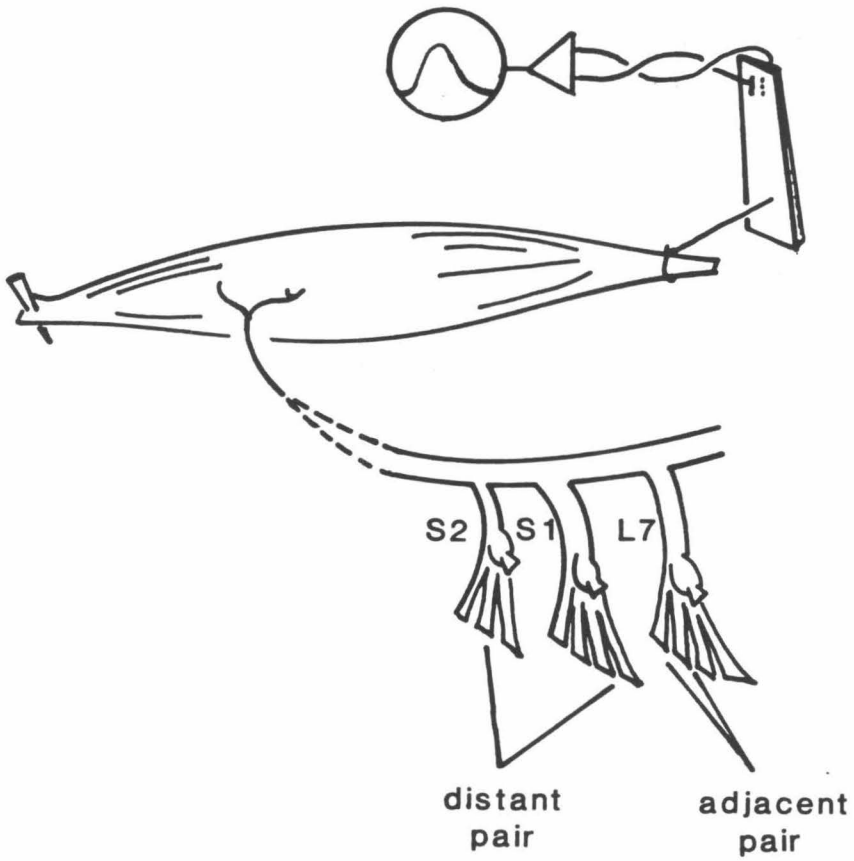



Figure 2

If we treat a multiunit filament as a single motor unit, and if the tension generated by stimulating each filament is 25%, then the estimated overlap between two such filaments in the absence of topography would be 25%.

In these experiments, we compared overlap between adjacent and distant pairs of ventral root filaments. In a topographically innervated muscle, adjacent pairs of filaments should have greater tension overlap than distant pairs, while in a muscle that lacks topography there should be no systematic difference between adjacent and distant pair measurements. To obtain overlap measurements, we selected ventral root filaments whose positions in the spinal cord were either adjacent or distant (Figure 2). Adjacent pairs of filaments were chosen from a single ventral root. To select pairs of distant filaments, ventral root S1 was divided into 4 or 5 filaments, and ventral roots L7 or S2 were divided into two if they contributed more than about 40% of the soleus tension. Distant filaments always had at least 3 intervening filaments, and were usually from different ventral roots. For example, if one of the pair was the rostralmost quarter of S1 then the other might be the caudal half of S2. Overlap measurements were made using tetanic tension, since in preliminary experiments it gave more consistent measurements. In addition, tetanic tension measurements reduce the tension nonlinearity produced by the series elastic component of muscle (Brown and Matthews, 1960). We stimulated filaments at 40 Hz for 600-800 ms, at 7 - 10 volts (about 1.5 times threshold). For a given

overlap measurement, filaments were stimulated separately and together in a pseudorandom order. Each stimulus train was followed by a rest interval of at least two minutes. At least three sets of measurements were taken for each pair of filaments. Because of the proximity of the suction electrodes, we tested for current spread by applying voltage when a filament was just outside the electrode tip and confirmed that applied voltages in this configuration did not elicit muscle contraction. Ten distant pairs and ten adjacent pairs were used in this comparison. In addition, we measured overlap between filaments in 13-15 day rabbit soleus, in which synapse elimination is virtually complete, in order to control for tension overlap that is not related to polyinnervation.

*Retrograde labeling of motor neurons.*

We retrogradely labeled spinal cord motor neurons following intramuscular injection of horseradish peroxidase conjugated to wheat germ agglutinin (HRP-WGA, Sigma), in a 5% solution in distilled water. Animals were anesthetized with inhaled metofane (Pitman-Moore) followed by ethyl ether. The soleus muscle on one side was exposed following a lateral incision in the posterior hindlimb, through the lateral gastrocnemius. The muscle was held away from surrounding muscles with a blunt, curved glass probe, and a small piece of cotton was placed beneath the probe to minimize leakage of HRP-WGA into neighboring muscles. Both soleus muscles were injected in

each rabbit. On one side, 0.25 µl of HRP-WGA was injected through a Hamilton syringe oriented parallel to the long axis of the muscle, in order to keep the injection restricted to a local portion of the muscle. On the contralateral soleus within the same animal, two or three injections of 0.5 µl each were made, or else a single injection of 0.75 µl, in order to label the entire soleus muscle. In most cases a small piece of moistened Gelfoam (Upjohn) was placed on the injection site to minimize leakage of HRP-WGA into neighboring muscles. The nerve entry point into the muscle and the distal tendon were used as landmarks so that injections could be directed at endplate-rich areas. After recovery from anesthesia, animals were returned to their hutches.

After a two day survival to allow for transport of HRP-WGA, animals were deeply anesthetized and perfused intracardially with saline followed by 4% paraformaldehyde. The spinal cords were marked with pinholes and nicks to verify orientation and allow for alignment of sections during reconstruction. Spinal cords and muscles (including the soleus muscle and all neighboring muscles dorsal to the tibia) were dissected and allowed to equilibrate in 20% or 30% sucrose in phosphate buffer. In some cases, additional fixation was required and the tissue was equilibrated in a sucrose/paraformaldehyde solution.

Spinal cords were sectioned longitudinally on a freezing microtome at 60, 90, or 100 μm. Muscles were frozen quickly in isobutane cooled with dry ice. They were cut into 40 μm transverse sections on a cryostat and mounted directly onto gelatin coated slides. The relative positions of the soleus and neighboring muscles were thus preserved throughout tissue processing. Floating sections of spinal cord and slide-mounted sections of muscle were processed to reveal HRP-WGA label using the tetramethylbenzidine (TMB) reaction (Mesulam, 1978).

Muscle sections were evaluated to determine the extent of label on each side, and to ensure that a minimal amount of label could be detected in neighboring muscles. Animals were accepted for further study if no label was visible in muscles neighboring the soleus or at most it was limited to the margin around the muscles, and if the whole soleus was labeled on one side, but only part (2/3 or less, usually less than 1/2) of the soleus was labeled on the other side.

Spinal cord sections containing retrogradely labeled cell bodies were traced and scored on an IBM PC using customized software written by James Knierim. Stacks of sections were collected for each spinal cord. These were aligned and analyzed on an IRIS 2400 computer (Silicon Graphics) using customized software written by David Bilitch. Both sides of the spinal cord were divided into 10 rostrocaudal positional bins based on the beginning and

end of the completely labeled motor pool, where bin 1 was the most caudal and bin 10 was the most rostral. The cells in each bin were counted on both sides. This procedure gave a pair of spatial distribution histograms for each spinal cord.

## Results

*Intracellular recording*

In these experiments, we examined the relationship between the positions of motor neurons within a motor pool and the regions of the muscle they innervate. If the muscle were topographically innervated, the dorsal and ventral regions of the muscle should receive innervation from different parts of the motor pool.

Five animals in the early age group and 3 animals in the intermediate age group were included in this part of the study. The results are summarized in Figure 3. In the early age group, the mean index (see methods) was 56.5 $\pm$ 1.8 (s.d.) for the dorsal region and 52.4 $\pm$ 2.6 for the ventral region. These two values were not significantly different using unpaired ($p = .23$) or paired ($p =$

Figure 3. Histograms showing the mean innervation index for dorsal and ventral regions of the soleus muscle. Dorsal and ventral regions do not differ in their innervation indices.

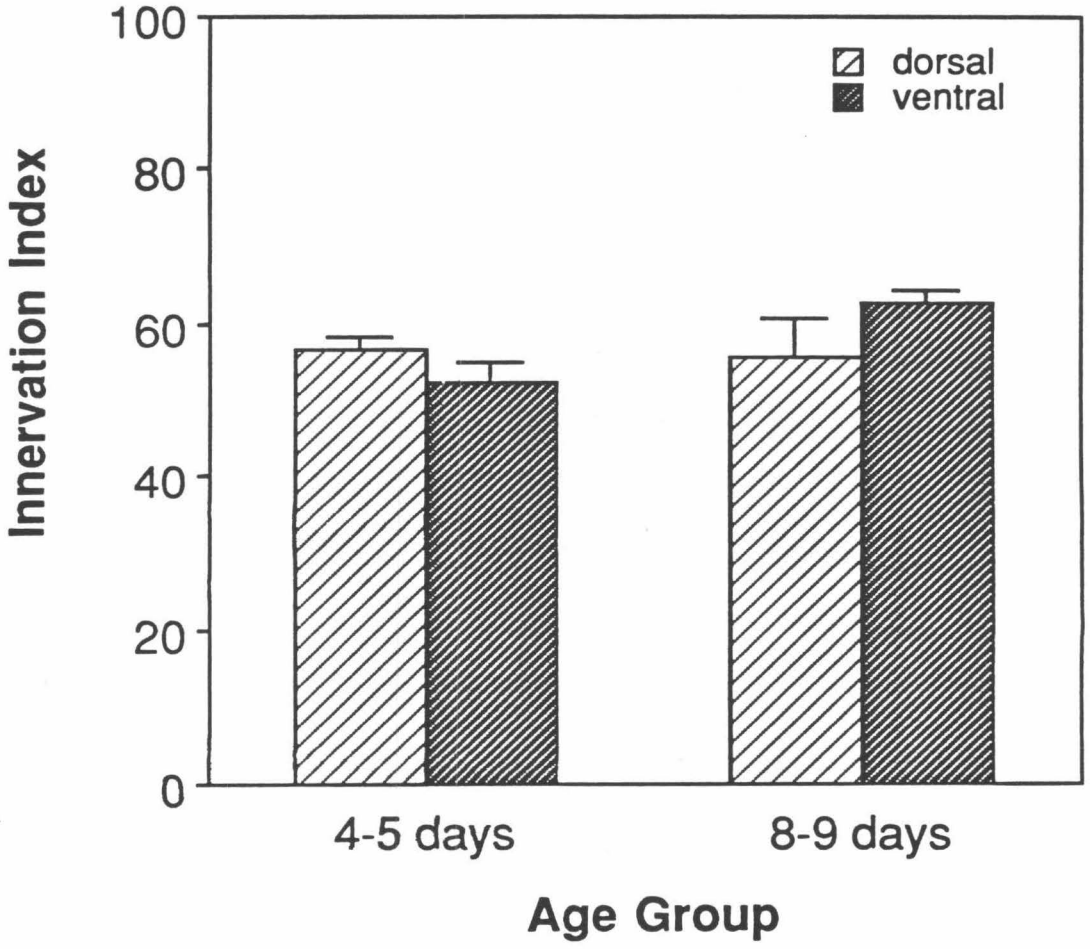

100
80
60
40
20
0
Innervation Index
dorsal
ventral
4-5 days
8-9 days
Age Group

Figure 3

.07) t-tests. At the intermediate age, the mean index was 55.5 $\pm$ 5.0 for the dorsal region and 62.6 $\pm$ 1.8 for the ventral region. In all cases, the indices for dorsal and ventral regions differed by less than 10.

While the above analysis gives an indication of the average positional origin of innervation to a particular region of muscle, an analysis of innervation to individual muscle fibers provides further details on the pattern of the projection. In a topographically innervated muscle, multiple inputs to a particular muscle fiber should originate in adjacent filaments more frequently than in distant filaments. Distant filament pairs were defined as those that had at least three intervening filaments. Given an arrangement of 5 or 6 filaments, there are more combinations of adacent filaments than distant filaments. In an arrangement of 5 filaments there are 4 combinations of adjacent filaments and 1 combination of distant filaments; in an arrangement of 6 filaments there are 5 combinations of adjacent filaments and 3 combinations of distant filaments. Thus, random (non-topographic) multiple innervation would have an apparent bias for adjacent filaments by 4:1 for 5 total filaments, or 5:3 (or 1.7: 1) for 6 total filaments. A bias more pronounced than these chance ratios would be indicative of a topographic projection to the muscle.

We compared the actual occurrence of adjacent and distant filaments to their predicted occurrence based on random innervation. In preparations arranged into 5 filaments, there were 66 pairs from adjacent filaments, and 17

pairs from distant filaments. These pairs of inputs are in a ratio of 3.9:1. In preparations that included 6 filaments, there were 48 pairs of inputs to individual muscle fibers that originated in adjacent filaments, and 33 pairs that originated in distant filaments; here the ratio is 1.5:1. In both of these groups, the relative occurrence of adjacent and distant inputs at individual muscle fibers is similar to that predicted for random, non-topographic innervation.

*Tension overlap.*

While the intracellular recordings allowed examination of the origin of inputs at individual muscle fibers, the method is limited in that the muscle fiber sample size from each preparation is relatively small. Using the tension overlap method, the innervation of large numbers of muscle fibers can be examined at once.

A typical overlap measurement is shown in Figure 4. Fluctuations in these measurements were minimized by allowing time to recover after tetanic stimulation, and by averaging overlap measurements over at least 3 trials. The standard deviation in a set of repeated tetanic tension measurements was typically less than 10% of the mean.

Figure 4. An example of a typical tetanic tension overlap measurement obtained in this experiment. The overlap in this example is 40%.

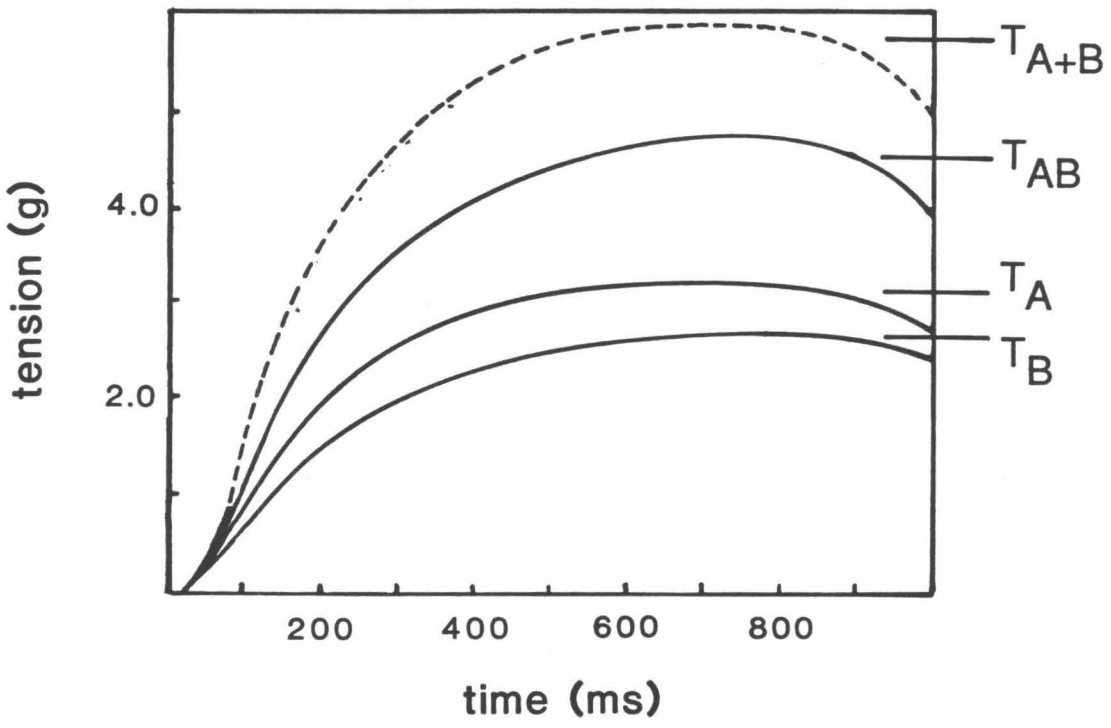



Figure 4

We made 10 overlap measurements for each of the two experimental groups, where one measurement represents tension overlap between two filaments. The average tension overlap between pairs of adjacent filaments (±s.e.m.) was 44 ± 2.8%. For distant pairs, the average overlap was 41 ± 2.4%. For the 20 filaments included in measurements on adjacent pairs, the overall mean size was 29 ± 1.3% of the maximum twitch tension of the muscle. The mean size of filaments used in distant pair measurements was 28 ± 1.2%. Results from these experiments are summarized in Figure 5. Comparison of tension overlap for adjacent pairs to tension overlap for distant pairs, using analysis of covariance with size as a covariate, revealed that the means were not significantly different ($p = .54$). Hence, adjacent and distant pairs of filaments have indistinguishable degrees of tension overlap.

In order to assess how much of the tension overlap in the experimental groups was due to polyinnervation, we measured tension overlap between ventral root filaments in singly innervated soleus muscles as a control. Tension overlap in these muscles is due to something other than polyinnervation, and is possibly a result of mechanical features of the muscle. We used 10 pairs of filaments from animals in the late (13-15 day) age group. The mean tension overlap for pairs of filaments at this age was 23 ± 3.5%. The mean size of these filaments was 24 ± 1.9% of the maximum twitch tension of the muscle. Overlap values for this age group were compared to overlap values for the

early age group in which adjacent and distant pairs were pooled together. The mean overlap for all pairs in the early age group was 43 $\pm$ 1.8%; filaments used in these measurements had a mean size of 28 $\pm$ 0.9%. Analysis of covariance was used to compare overlap at the early age to overlap at the late age, and, as in the comparison between adjacent and distant pairs, filament size was a covariate. This statistical test allowed comparison of the mean overlap values between the two groups and remove any effect that filament size might contribute to mean overlap. It was important to remove the effect of filament size because the mean filament size was greater in the early age group than in the late age group. After correction for this effect, the tension overlap in the early age group was significantly greater than tension overlap in the late age group ($p < .001$). Thus, it is likely that tension overlap between two filaments is indicative of shared muscle fibers during polyinnervation, albeit over a nonzero baseline.

The ventral root filaments used to determine tension overlap were selected on the basis of their size and rostrocaudal position. Because of the need for large filaments, the filaments contained several adjacent motor units, and thus included both fast and slow motor axons. It is possible, therefore, that overlap values would depend on the similarity or difference in motor unit composition between the two filaments in a pair. We tested for this effect by comparing the difference in twitch rise times of the filaments (normalized to the

twitch rise time of the whole muscle) with the tension overlap between them. We defined a rise time difference (rtd):

$$(8) \qquad rtd = (RT_A - RT_B)/(RT_M)$$

where $RT_A$ and $RT_B$ are the twitch rise times of the two filaments, and $RT_M$ is the twitch rise time for the whole muscle. When rtd was included as a covariate in an analysis of covariance, rtd did not vary with overlap ($p > .2$). Thus pairs of filaments with large differences in twitch rise times can have as much tension overlap as pairs of filaments with very similar rise times. This finding does not necessarily imply a lack of specificity in innervation of fiber types. Rather, the lack of correlation may be a consequence of the fact that at four days, motor unit twitch rise times, especially for slow motor units, have a wide range. Rise times for fast motor units have a mean of 227 ms and range from 192 ms to 250 ms; rise times for slow motor units have a mean of 430 ms and range from 300 ms to 597 ms (see Chapter 3). Two motor units can have a large difference in rise time (e.g., 200ms) and be the same type (slow), or a small difference in rise time (50-100ms) and be different types.

Figure 5. Histograms showing distributions of percent overlap for adjacent (A), distant (B), and control (C) pairs of ventral root filaments. Arrows indicate means for each distribution. Overlap between adjacent and distant filaments was not significantly different from each other ($p = .54$, analysis of covariance with filament size as covariate). The control group consists of overlap measurements made on 13-15 day animals, in which synapse elimination is mostly complete, and in which tension overlap due to polyinnervation should be minimal. Both the adjacent and distant filament groups from 4-5 day animals differed significantly from the older controls ($p < 0.001$). These results suggest that the proximity of motor neurons in the spinal cord does not determine the extent to which motor units share muscle fibers during polyinnervation.

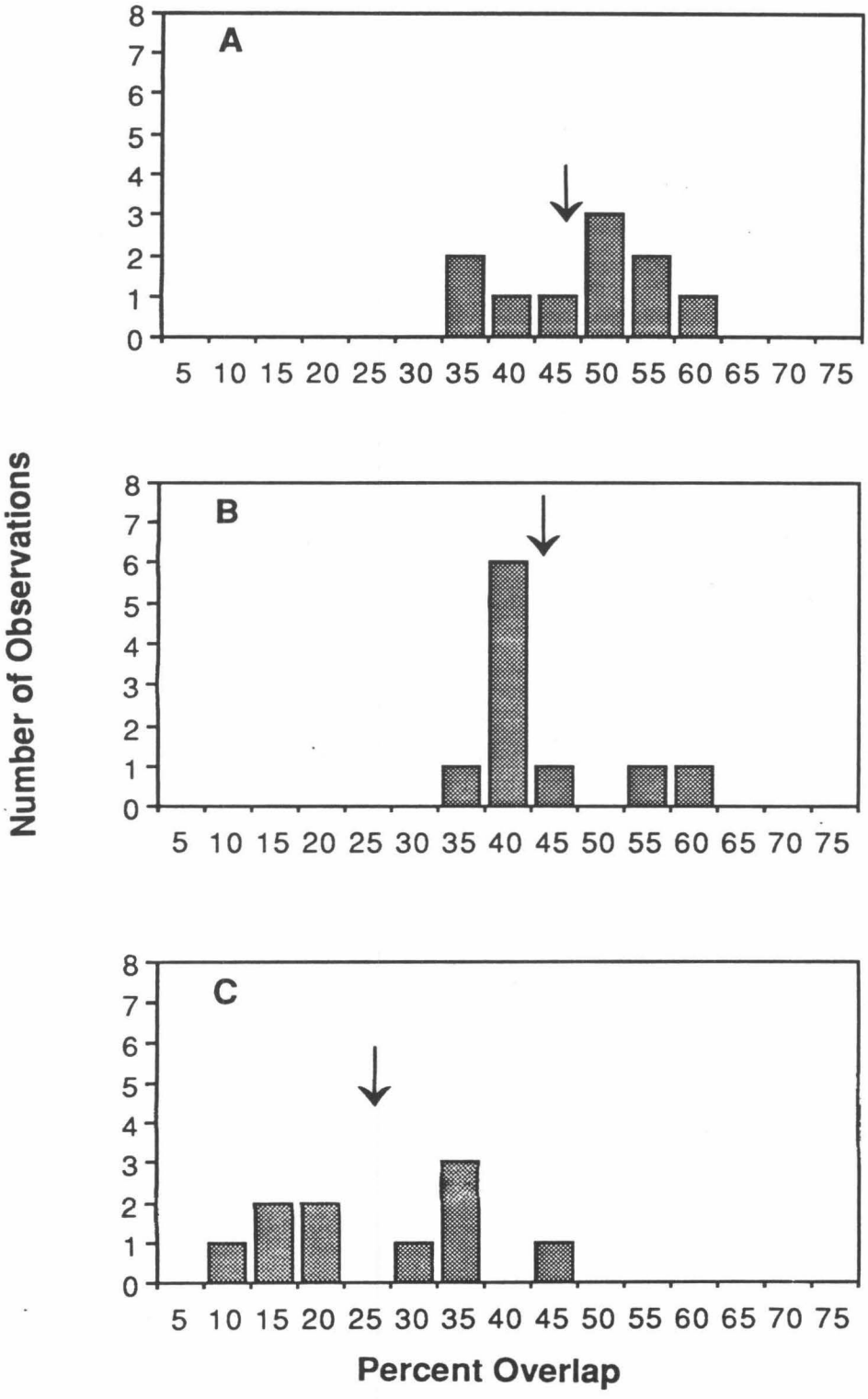
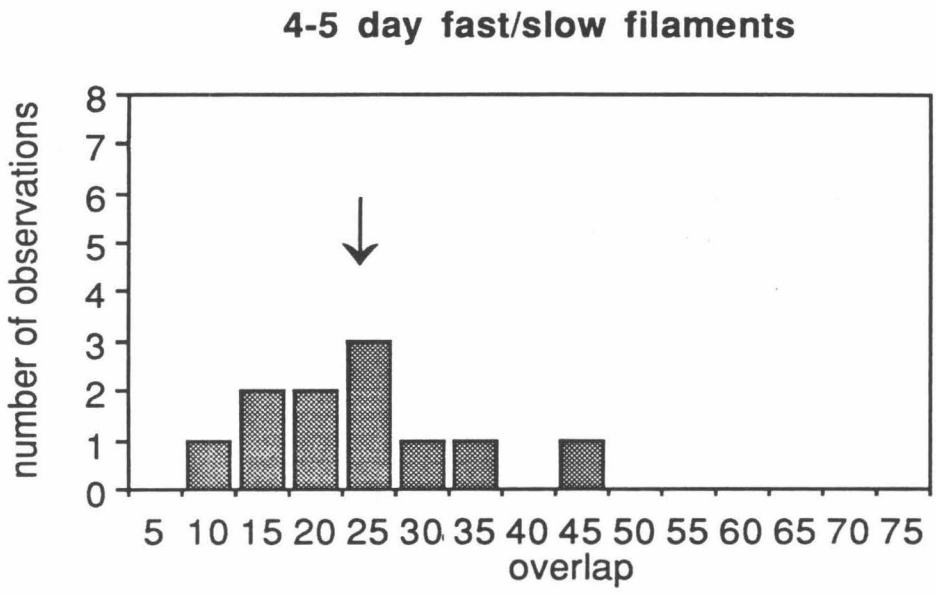
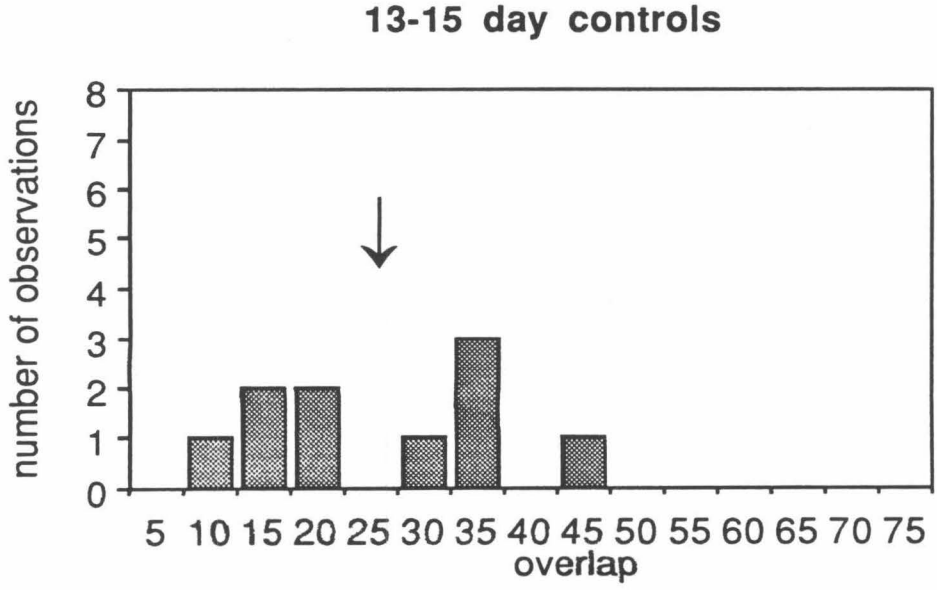

A
B
C
Number of Observations
0 1 2 3 4 5 6 7 8
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
Percent Overlap

Figure 6. Histograms showing distributions of percent overlap from measurements in which one filament contains all fast motor units and the other contains all slow motor units. These measurements do not differ from the singly innervated controls ($p = 0.3$), shown below. Arrows indicate mean overlap values.


4-5 day fast/slow filaments
number of observations
0
1
2
3
4
5
6
7
8
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
overlap


13-15 day controls
number of observations
0
1
2
3
4
5
6
7
8
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
overlap

To specifically address overlap between fast and slow motor units, we measured overlap between multiunit filaments where one filament contained only slow units and the other only fast units (Figure 6). The mean overlap for this group was 20.6 $\pm$ 2.9% and the average size of the filaments used was 15.6 $\pm$ 1.1%, n = 11. Using an analysis of covariance to remove the effect of filament size, we found that the tension overlap between these filaments was significantly less than the tension overlap we found in the experimental group, where the filaments contained a random mixture of fast and slow motor units ($p < 0.05$). Furthermore, this tension overlap did not differ from that of the singly innervated controls ($p = 0.30$). Thus, our results from these tension overlap measurements are consistent with previous findings (Thompson et al., 1984; Gordon and Van Essen, 1985) that connections between motor neurons and fast or slow muscle fibers are specific.

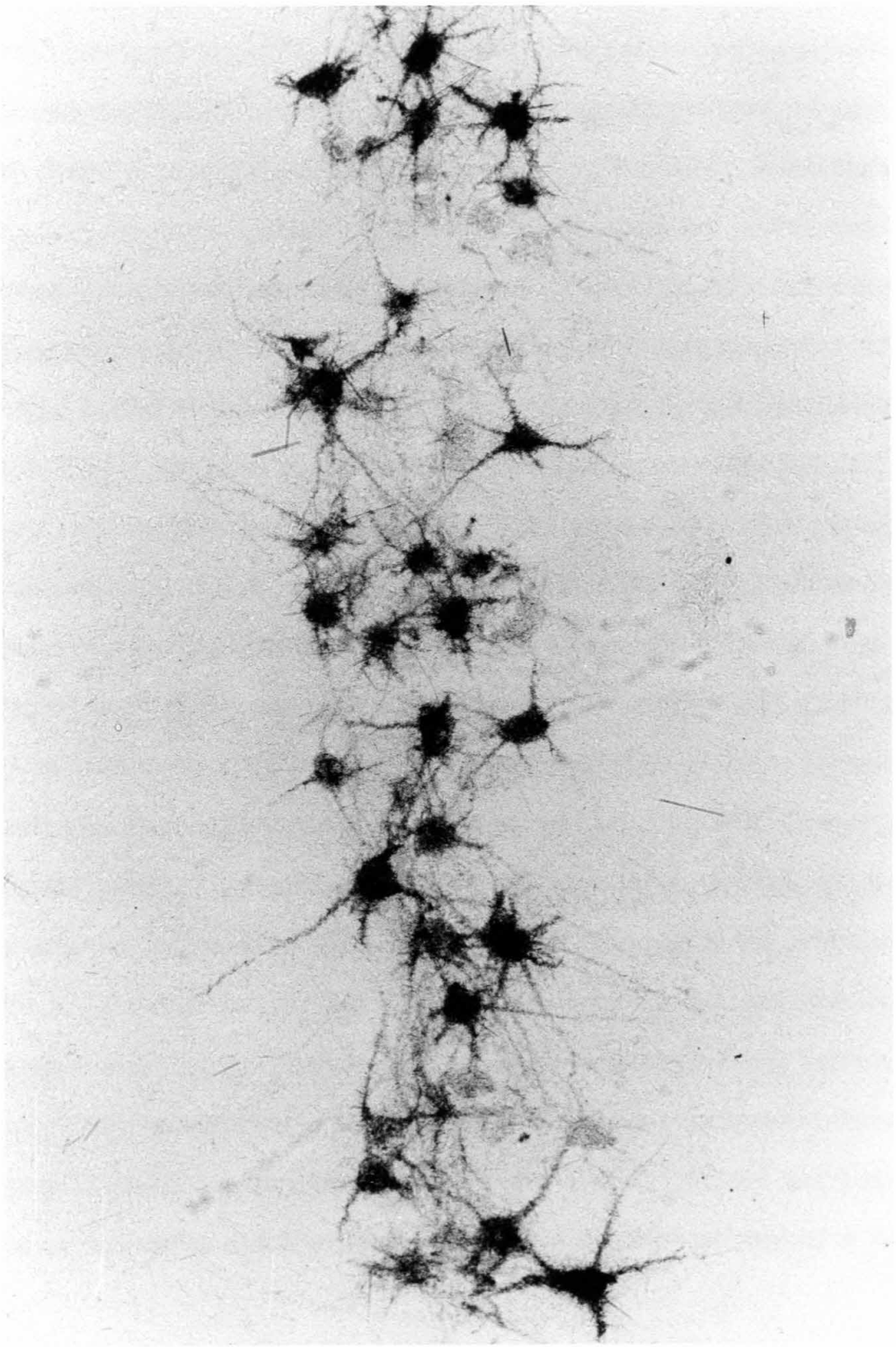
*Retrograde labeling of motor neurons.*

Tension overlap experiments to study topography are meaningful only during polyinnervation, when muscle fibers can receive inputs from more than one motor neuron. In order to assess topography in older animals which have undergone synapse elimination, and to confirm our findings for younger animals, we used anatomical methods which do not rely on polyinnervation. In these experiments, we injected a retrograde tracer into restricted portions of

muscles, then examined the positions of motor neurons which were labeled in the spinal cord. If the projection from the spinal cord to the muscle were topographic, then small muscle injections would label spatially restricted subsets of motor neurons within the motor pool. Moreover, we would expect a correlation between the location of the injected muscle region and the location of the subsequently labeled motor neurons. Conversely, if the projection were not topographic, then small muscle injections would label diffuse subsets of motor neurons in the spinal cord, and we would expect no correlation between the location of the injected muscle region and the location of the labeled motor neurons.

Following injections of muscle, we visualized retrogradely labeled neurons in the spinal cord (Figure 7). Labeled cells varied in staining intensity. In spinal cords sectioned at 60 μm, many labeled cells had visible dendrites, although some lightly labeled cells had few or none. When spinal cords were cut into thicker sections (90 or 100 μm), the staining appeared more uniform and most labeled motor neurons had visible dendrites. All labeled motor neurons in sections of either thickness were scored and counted. After correcting for double counting (Abercrombie, 1946), we counted the cells on the side where the whole motor pool was labeled. The average number of cells for

Figure 7. Longitudinal section of a spinal cord showing motor neurons labeled by ipsilateral injection of the soleus muscle with HRP-WGA.



soleus motor pools was 123$\pm$ 57 (s.d., n = 15). Eccles and Sherrington (1930) estimated that 33% of all motor axons are gamma motor neurons. Another study (Burke et al., 1977), estimated that between 24.6 and 29% of the motor neurons in cat medial gastrocnemius and soleus muscles are gamma motor neurons. Using these figures as a guideline, we estimate that the soleus motor pool contains about 80 to 90 alpha motor neurons, which is in agreement with previous studies of the rabbit soleus motor pool (Gordon and Van Essen, 1985). Two of the spinal cords used in the study had greater than 200 motor neurons labeled on the side corresponding to the whole muscle injection; one of these was greater than two standard deviations from the mean. Although the muscles surrounding the soleus did not appear extensively labeled in our sectioned material, there may have been some uptake of HRP-WGA by motor neurons in portions of muscles outside the sectioned region in these animals. Specifically, these regions would be in the ventral part of the limb, as ventral muscles were not included in sectioning blocks, or in regions of the gastrocnemius or lateral plantaris muscles rostral or caudal to the sectioned portions. The former possibility is unlikely, since ventral muscles were not exposed during surgery. However, the latter possibility remains likely because some of the connective tissue surrounding these muscles was disrupted during surgery. Furthermore, our sections only included a region of about 4 or 5 mm centered around the endplate region of the soleus; the endplate regions of the

surrounding muscles may lie in regions outside these 4 or 5 mm, and would thus not be included in the sections. Removing these two animals with greater than 200 motor neurons labeled does not alter the results we obtained in the analysis below.

On the contralateral side, which received local tracer injections, the extent and position of the injections were quantified using camera lucida tracings of muscle sections (Figure 7). In several cases traced muscle sections were reconstructed, and it was verified that the position of the label was consistent through the sectioned region. To quantify the extent of the injection, a line was drawn from the center of the muscle to each border of the traced region. The arc subtended by these two lines containing the label was defined as the angular extent of the injection, $\theta$. To determine the position of the injection site within the muscle, a line from the center of the muscle to the ventrolateral corner was used as a reference because it was often the easiest to identify based on the relative positions of neighboring muscles in the section (Figure 7). We measured the angle subtended by this reference line and a line bisecting the labeled area with increasing angle corresponding to moving dorsally from the reference line. This angle, $\phi$, was defined as the position of the injection.

Figure 8. Camera lucida drawings of left and right muscle sections of a 4-day old rabbit used in this study. Sol, soleus muscle. In the upper panel, the positions of the local injection in the left muscle is $245^{o}$; the extent is $60^{o}$.

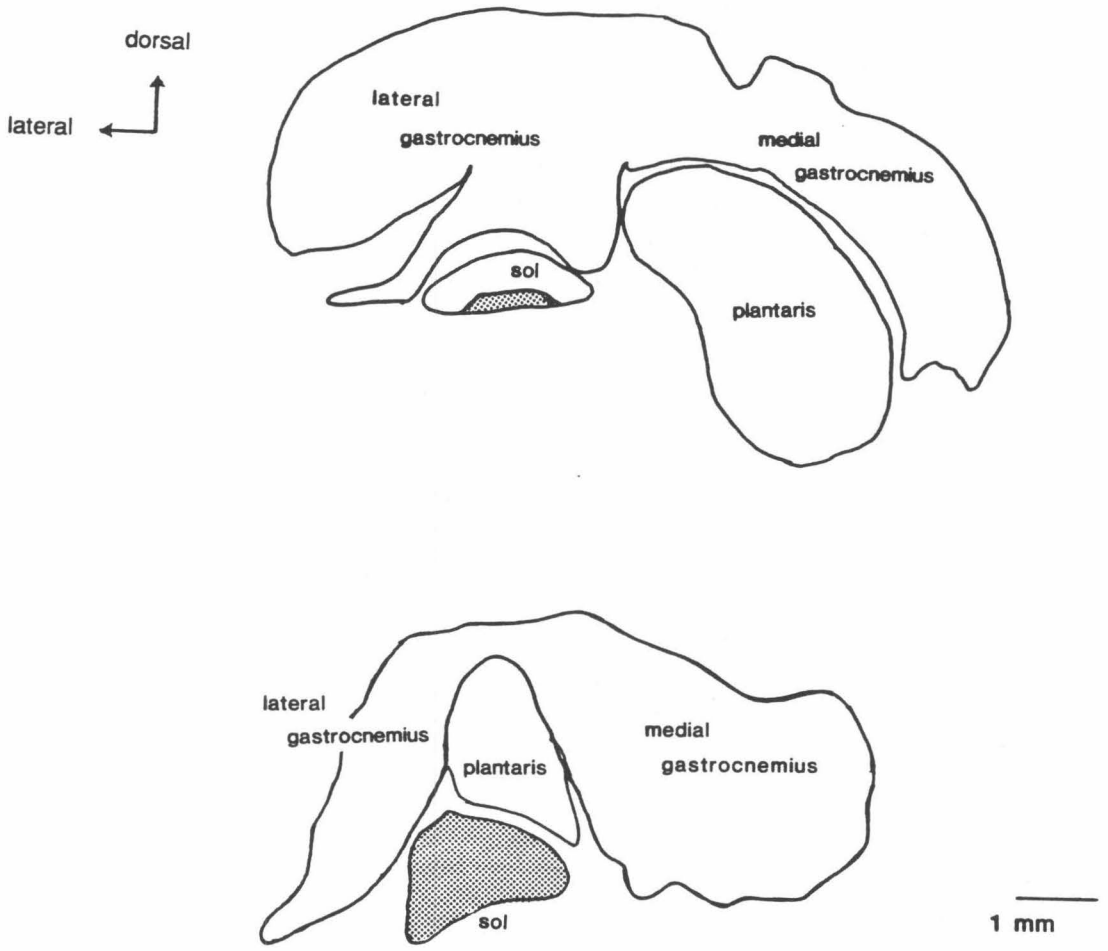

dorsal
lateral
lateral
gastrocnemius
medial
gastrocnemius
sol
plantaris
lateral
gastrocnemius
plantaris
medial
gastrocnemius
sol
1 mm

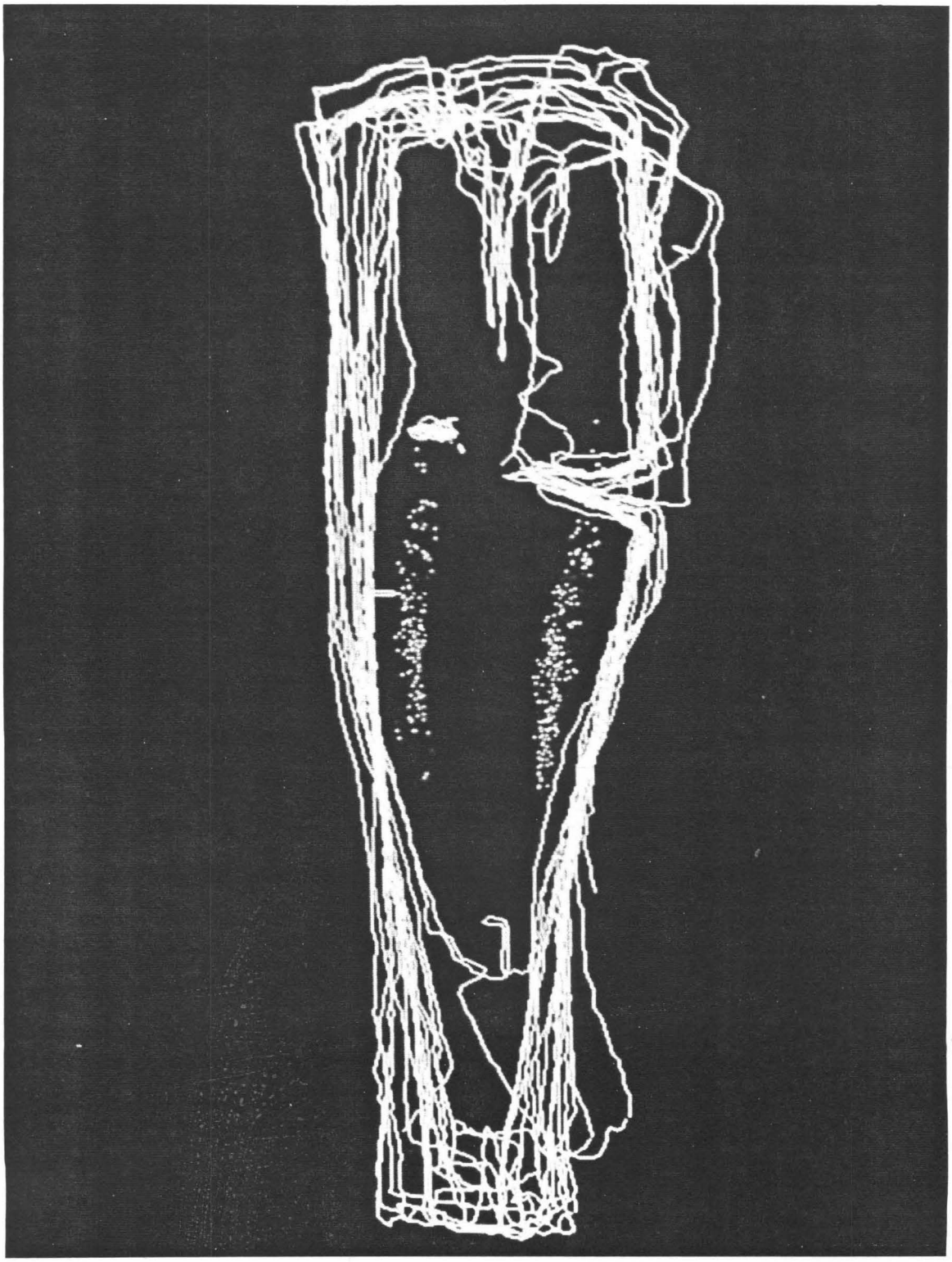
Figure 8

To quantify the position and clustering of labeled groups of motor neurons, we used computer-generated spatial distribution histograms from reconstructed spinal cords (Figure 9). Because the motor pool is long and narrow, we took into account only the rostrocaudal position of motor neuron cell bodies. Each spinal cord had two distributions: one for the locally injected side and one for the whole motor pool, which we used as a reference. Each distribution had a mean rostrocaudal position and a standard deviation. The standard deviation of the mean motor neuron position on the locally injected side, $\sigma$, was used to quantify clustering of motor neurons in the spinal cord. The position within the motor pool of motor neurons labeled following local muscle injections was defined as follows:

(9) $$\Delta m = m_{tot} - m_{loc}$$

where $m_{tot}$ = the mean position of the completely injected side and $m_{loc}$ = the mean position on the locally injected side in units of positional bins. The absolute value of $\Delta m$ is a measure of the shift in the mean position of motor neurons on the locally injected side with respect to the mean position of the whole motor pool. Positive and negative values of $\Delta m$ indicate caudal and rostral shifts, respectively.

Figure 9. Following injections, the label in the spinal cord was examined, and longitudinal sections of spinal cord were scored and reconstructed. The left shows label following a local injection in the left soleus; the right shows the whole soleus motor pool.



Three animals from the early age group and 12 animals from the late age group met our criteria and were included in the anatomical study. For the analyses described here, we pooled data from both age groups and used multiple regressions in which the effect of age on a given parameter could be assessed. Age had no effect on any of the correlations ($p > .5$).

To determine whether small injections label clustered groups of motor neurons, we examined the correlation between the extent of the muscle injection and the clustering of the labeled motor neurons in the spinal cord (Figure 10). A linear regression of $\sigma$ with $\theta$ showed no correlation ($p > 0.10$), indicating that local regions of muscle are innervated by motor neurons from a broad sampling of rostrocaudal positions. This lack of clustering contrasts with what would be expected if the projection to the muscle were topographically organized. We also assessed whether or not there is a mapping between different regions of muscle and the rostrocaudal positions of motor neurons innervating these regions (Figure 11). A linear regression of $\Delta m$ with the position of muscle label, $\phi$, showed no correlation ($p = 0.5$). The absence of a correlation indicates that neighboring regions of the motor pool do not consistently innervate neighboring regions of muscle.

Figure 10. Scatter plot showing no correlation between the extent of label in the muscle and the standard deviation of the mean position of cells following local injection.

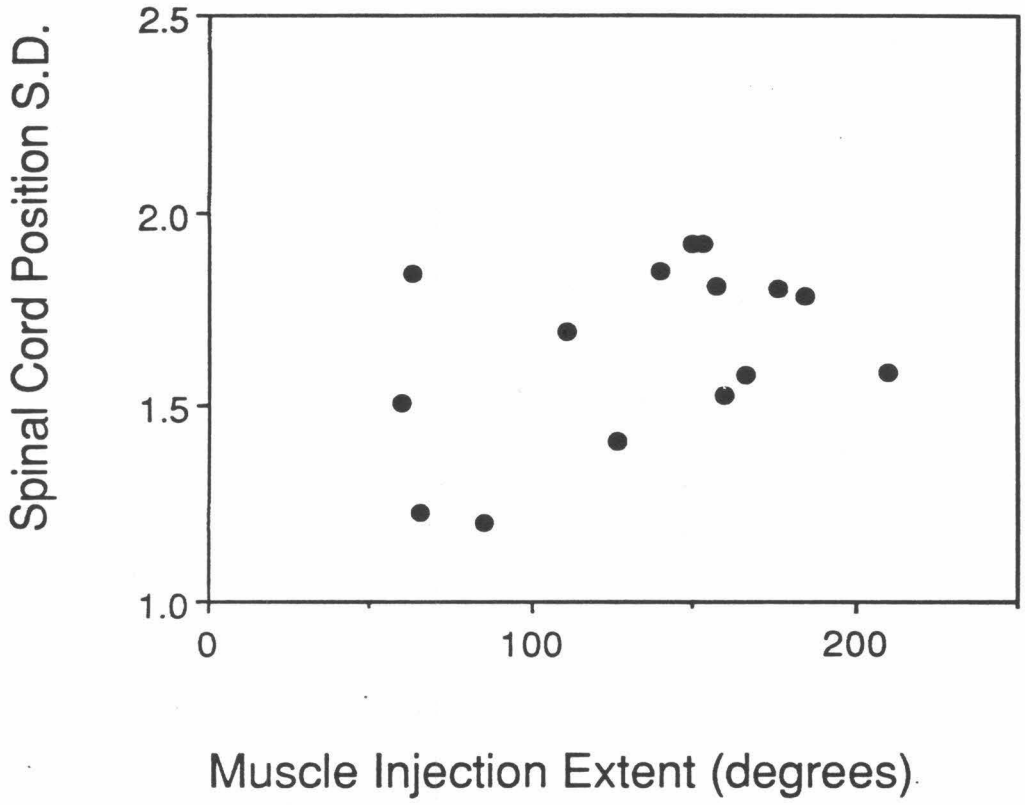

Spinal Cord Position S.D.
2.5
2.0
1.5
1.0
0
100
200
Muscle Injection Extent (degrees)

Figure 10

Figure 11. Scatter plot of the position of the local injection in the muscle vs the mean position of retrogradely labeled cells with respect to mean position of cells in the whole motor pool; no correlation is observed.

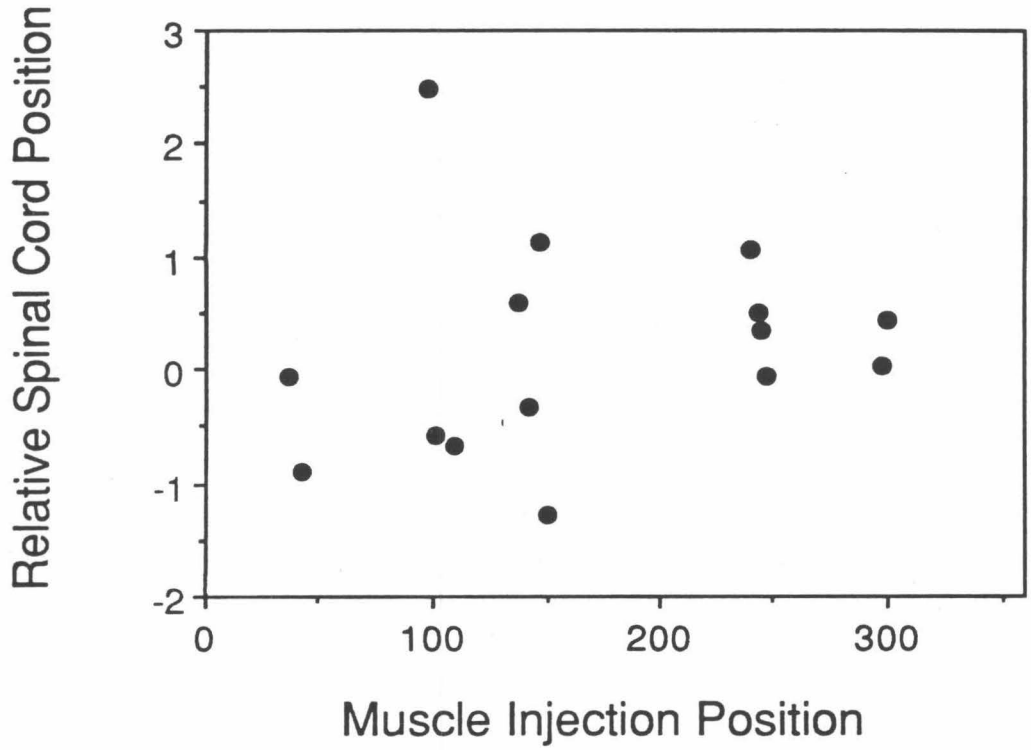

Relative Spinal Cord Position
3
2
1
0
-1
-2
0
100
200
300
Muscle Injection Position

Figure 11

**Discussion**

In this study, we have examined the topographic organization of innervation to the rabbit soleus muscle before and after postnatal synapse elimination. Using intracellular recording, we found no difference in the rostrocaudal positions of spinal cord motor neurons innervating dorsal versus ventral regions of the muscle. This contrasts with results from studies which have used a similar method to assess topography in other muscles. In the rat serratus anterior and diaphragm muscles, different regions of the muscle have a bias toward innervation from different spinal roots (Laskowski and Sanes, 1987). These muscle regions are organized into rostrocaudal sectors, and thus the axis of topography seems intuitive. In the soleus muscle, however, no obvious anatomical subdivisions are evident. We examined only the dorsoventral axis, because it can be reliably exposed in an intracellular recording preparation. It remains possible that a difference in the innervation bias was missed because it occurs along a different axis from the one examined.

We also examined the positions of ventral root filaments providing inputs to individual polyinnervated muscle fibers. Innervation of individual polyinnervated muscle fibers did not originate in adjacent pairs of filaments more often than expected for random, non-topographic innervation. Such a

bias would be expected if neighboring motor neurons innervated neighboring regions of muscle. Furthermore, this lack of selectivity was evident at both the ages we examined, and thus it is unlikely that synapse elimination plays a role in sharpening topographic selectivity in the soleus.

The intracellular recording method was advantageous in several respects: it allowed direct examination of shared innervation at the level of individual endplates; it was possible to include an analysis of all possible pairings of ventral root filaments within a single muscle; it was possible to examine the innervation of two different regions of the muscle in a single preparation, whereas in the retrograde labeling experiments it was only possible to look at one region within each muscle; and because all of the innervation remained intact during the recordings, revealing finer detail about the rostrocaudal position of multiple inputs to single muscle fibers. However, using intracellular recording, only a relatively small number of muscle fibers can be sampled from a single preparation.

Tension overlap comparisons allowed examination of shared innervation in a large sample of muscle fibers. We found no difference in the tension overlap produced by stimulation of adjacent versus distant pairs of ventral root filaments, consistent with our result that synapses at an endplate do not prefentially arise from neighboring regions of the spinal cord. The tension overlap from adjacent and distant ventral root filaments was compared to that

found in two control groups. In one of these groups, tension overlap was measured in muscles from older animals, in which synapse elimination is complete. Because, by definition, motor units cannot share muscle fibers in singly innervated muscle, there should be no tension overlap in this group. Although we did find some tension overlap in the older muscles, the overlap was significantly less than that found in polyinnervated muscle (see below). In the second control group, tension overlap was measured following stimulation of a pair of filaments where one filament contained only fast motor units and the other contained only slow motor units. This tension overlap would be less than that measured following stimulation of filaments containing a random mixture of fast and slow motor units if connections to fast and slow muscle fibers were specific. Tension overlap between all-fast and all-slow filaments from polyinnervated muscles was indeed significantly less than overlap between mixed filaments, and was comparable to the overlap found in singly innervated control muscles.

Our results from these experiments on fast vs. slow specificity illustrate that the tension overlap method can reveal specificity of connections. It is thus likely that this method would have revealed large differences (e.g., twofold) between adjacent and distant pairs of ventral root filaments if the projection had marked topography. That there was no difference supports our other evidence that, in the polyinnervated soleus, there is little, if any topography.

While these controls support the view that tension overlap is an indication of shared innervation, both homogenous fast or slow filaments and filaments from singly innervated muscle still had substantial tension overlap. Moreover, the tension overlap in the polyinnervated muscles was about twice as high as our estimates predicted. It is thus necessary to consider sources of tension overlap distinct from polyinnervation. These sources may be acting on measurements from both age groups, and may be related to mechanical aspects of muscle contraction. For example, Brown and Matthews (1960) have noted that a series elastic component of muscles, common to both filaments, may give an artificially high tension overlap. This effect was greatly reduced when tetanic tension was measured rather than twitch tension, although it may remain to some extent. Another possible source of error is current spread from one filament to another. This effect can be minimized by stimulating with voltages close to threshold (Brown and Matthews, 1960). In these experiments, filaments were stimulated using voltages that were about 1.5 times the threshold voltage. Furthermore, we could not elicit contraction by stimulating when a filament was outside the electrode. For these reasons, it is unlikely that current spread was a major source of error. A third possibility is that mechanical coupling between muscle fibers may give rise to nonlinear tension summation. Such coupling may depend on the number of muscle fibers contracting at once. Powers and Binder (1991) have examined the

summation of single motor unit tension in the cat tibialis posterior muscle. In this muscle, single motor unit tensions have greater than linear tension summation. This nonlinearity is reduced when small, trapezoidal movements are introduced during stimulation, presumably because such movements reduce friction between contracting muscle fibers and nearby passive fibers. We found greater than linear summation (negative overlap) in some cases when stimulating single motor units (unpublished observations). Powers and Binder (1991) found that the amount of nonlinearity size did not vary with motor unit size; however, they did not report on comparisons involving groups of several motor units.

We confirmed these findings for polyinnervated muscle and extended them to singly innervated muscle using retrograde labeling of motor neurons. In a topographically innervated muscle, neighboring regions of muscle should be innervated by neighboring regions of the motor pool. Data from both age groups revealed no correlation between the positions of the tracer injections within the muscles and the positions of subsequently labeled neurons within the motor pools. Thus, there is no consistent mapping of spinal cord positions to positions within the muscle. Since each muscle received only one local injection, it remains possible that each soleus muscle is topographically innervated, but that the mapping varies among individuals. However, even if this were the case, small, local injections would label restricted groups of motor

neurons. Injection size did not correlate with standard deviation of the positions of labeled motor neurons within the spinal cord, indicating that small injections do not label tightly clustered groups of cells. Rather, any small region of muscle evidently receives innervation from motor neurons with a broad spatial distribution, consistent with a non-topographically organized projection. That our results from the retrograde labeling experiments are similar at the early and late age groups suggests that there is no improvement in topography as a result of synapse elimination.

The results described here suggest that the projection from the spinal cord to the rabbit soleus muscle lacks topography. Furthermore, we found no improvement of topography following synapse elimination, suggesting that refinement of topography is not a primary function of synapse elimination in the soleus muscle. In the rat lateral gastrocnemius muscle (Bennett and Lavidis,1984), and in the rat serratus anterior (Laskowski and Sanes, 1987; Laskowski and High, 1989), topography is significantly improved following removal of redundant synapses, implying a role for synapse elimination. However, unlike the soleus, the lateral gastrocnemius muscle has some measurable topography even before synapse elimination (Bennet and Lavidis 84). Perhaps neuromuscular synapse elimination has several roles, which are operative to different extents in different muscles.

A characteristic related to topography is muscle compartmentalization, in which the muscle fibers innervated by axons of primary nerve branches are restricted to particular regions of the muscle (English and Ledbetter, 1982; English and Weeks, 1984; Balice-Gordon and Thompson, 1988b). The primary nerve branch point is usually extramuscular in compartmentalized muscles, and there is evidence that, to some extent, the axons in these branches originate in separate parts of the motor pool (Balice-Gordon and Thompson, 1988b). In most cases, compartmentalized muscles have more than one origin or insertion. Frequently, compartments within a muscle are separated by fascial planes, such as tendinous sheets.

Unlike topographically innervated muscles, the soleus muscle is compact and has only two tendons. Furthermore, the lack of intramuscular fascial planes and an extramuscular nerve branch, and the asymmetry of primary nerve branches within the muscle, suggest that the soleus innervation is not divided into anatomically distinct compartments. English (1990) showed that in at least one compartmentalized muscle, axons do not branch at primary nerve branch points. In the soleus, preliminary observations suggest that some axons do in fact branch at these points (F. A. Williams, K. S. Cramer and D. C. Van Essen, unpublished observations). These axon branches would not be prevented from entering regions of muscle innervated by either of the primary branches, and thus it is unlikely that their synaptic connections would be

restricted to a muscle compartment.

Because compartmentalization and topography seem to correlate with certain anatomical features of muscles, it is likely that they are related to some aspect of functioning in those muscles. Topography in projections might allow for fine differential control over different regions of the muscle, and would thus be especially useful in muscles with distinct anatomical regions.

While the soleus muscle lacks topography, it is functionally partitioned in other ways. For example, the soleus contains a mosaic of fast and slow muscle fibers (Gordon and Van Essen, 1985) and fast and slow motor units have cell bodies distributed throughout the motor pool. These connections are specific (Thompson et al., 1984; Gordon and Van Essen, 1985) and divide the function of the muscle into different types of activation patterns. However, these functional groups are not anatomically partitioned in the neonatal rabbit soleus; rather, they are extensively interleaved in a uniform manner. Later in development, there is a gradient in the ratio of fast and slow muscle fibers across the muscle (Gordon, 1983). While this pattern suggests that different parts of the muscle are specialized for different types of contraction, our results on the lack of topography make it unlikely that there is a corresponding gradient in the ratio of motor neuron types within the spinal cord.

## CHAPTER 3:

# Maturation of fast and slow motor units during synapse elimination in the rabbit soleus muscle

## Introduction

Mammalian skeletal muscles contain distinct types of muscle fibers, which can be identified by a number of physiological and histological features. The two main classes are fast and slow, named because of their speed of contraction. The functional unit of muscle is the motor unit, which consists of a motor neuron and the collection of muscle fibers that it innervates. Motor units can also be classified as fast or slow, based on differences related to muscle fibers, such as contraction speeds and fatiguability, as well as on differences in neuronal properties, such as recruitment thresholds (Henneman, 1957), and conduction velocities (see Burke, 1981 for review). That fast and slow motor units can be distinguished physiologically suggests that fast and slow motor neurons are specifically connected to the corresponding muscle fiber types. Direct evidence for this specificity in adult muscles comes from histochemical studies of fiber types in glycogen depleted motor units (Edström and Kugelberg, 1968; Kugelberg, 1973), which showed that nearly all of the muscle fibers in each motor unit are of the same type. These findings present some interesting issues regarding the development of motor unit types. The first of these issues concerns the developmental mechanisms involved in establishing specificity in connections between motor neurons and muscle fibers. Because some of these mechanisms involve refinements of specificity (see Chapter 1), the degree of

specificity early in development is an important consideration in answering this question. A second question is how fast and slow motor unit properties emerge during development, and whether maturation proceeds differently in these motor unit types.

Neonatal muscles are polyinnervated and synapses are eliminated during the first few postnatal week until each muscle fiber receives a single input (Brown et al., 1976). Although there is considerable evidence that specificity is apparent early in development during polyinnervation, specificity in neonates is not as precise as that found in adults. In 8 day rat soleus, glycogen depletion studies have shown that 70% to 90% of the muscle fibers in a motor unit are one type; by 16 days, the fiber type composition of motor units exceeds 90% purity (Thompson et al., 1984). In neonatal mouse soleus, monoclonal antibodies specific for a given myosin isozyme labeled 50% to 70% of the muscle fibers in each unit; by two weeks this increased to nearly 100% (Fladby and Jansen, 1990). These results indicate that neuromuscular connections are somewhat specific in neonatal muscles, and that specificity improves later in postnatal development. Both neuronal specification of fiber types and selective elimination of inappropriate synapses may be involved in later refinement of motor unit specificity. The relative importance of different mechanisms may be related to the degree of specificity at different developmental stages. In the neonatal rabbit soleus muscle, the histogram of twitch tension rise times of

individual motor units is bimodal, which is suggestive of specific connections (Gordon and Van Essen, 1985), but the presence of motor units with intermediate rise times makes it difficult to assess the accuracy of specificity. It is not clear whether intermediate rise times reflect a population of intermediate axon and muscle fiber types, of fast and slow motor units whose rise times are at the high and low ends of their respective distributions, or of motor units containing a mixture of fast and slow muscle fibers. In this study, we reexamined the precision of specificity in the neonatal rabbit soleus muscle and estimated the extent to which axons make errors in their connections to muscle fibers.

Another important developmental consideration is the maturation of fast and slow motor unit properties. Some of the properties which distinguish motor unit types undergo changes during the period of postnatal synapse elimination; these changes can be different in fast and slow motor units. For example, it has been shown that motor unit twitch tension is larger for fast than for slow motor units in neonatal rabbit soleus (Gordon and Van Essen, 1985). While this tension, as a percentage of the total muscle tension, declines during the first two postnatal weeks in both fast and slow motor units, reflecting the loss of synapses, the decline is greater in slow motor units than in fast motor units (Gordon, 1983; Callaway et al., 1989). In this study, we have investigated further the maturation of motor unit properties. We measured twitch/tetanus

ratios in the two motor unit types at three developmental ages, and found that these ratios increase in fast motor units and decrease in slow motor units. Thus there are differences in the nature of changes which occur during the maturation of fast versus slow motor units.

The relative difference in the decline in twitch tension has been taken to suggest that slow muscle fibers receive more inputs than fast muscle fibers at the early ages (Callaway et al., 1989). Other evidence is consistent with this hypothesis. Estimates of the mean degree of polyinnervation in rat soleus, which contains mostly slow muscle fibers (Brown et al., 1976); are higher than those obtained for rat extensor digitorum longus (Balice-Gordon and Thompson, 1988a), which contains mostly fast muscle fibers. However, these studies did not distinguish between fast and slow muscle fibers within the muscles. Intracellular recording accompanied by lucifer yellow injections have been used to demonstrate that fast and slow muscle fibers are polyinnervated to the same extent at intermediate ages (Soha et al., 1987). Together, these findings suggest that synapse elimination occurs at different rates in fast versus slow motor units during the first week or so of synapse elimination, after which the rates become more similar. In the present study, polyinnervation by fiber type was reexamined in the early ages using estimates based on tetanic tension. In addition, because estimates based on twitch or tetanic tension are indirect, inputs at polyinnervated muscle fibers were also counted using

intracellular recordings of endplate potentials (e.p.p.'s) following separate stimulation of several ventral root filaments. Motor unit types in developing rabbit have principally been determined using the twitch tension rise times to peak following stimulation of axons in the ventral roots. In a preparation configured for intracellular recording, it is not feasible to record muscle tension. We therefore sought to distinguish axon types based on their e.p.p. latencies following ventral root stimulation, based on the fact that in adult soleus, fast motor axons have higher conduction velocities than slow motor axons (Bagust, 1974). There is some evidence that such differences are present in 2-week old cats (Bagust et al., 1974a); however, the distributions have not been examined during earlier development. This issue was investigated in rabbits of the early and intermediate age groups. We first showed, in a separate set of experiments, that fast and slow axons have different e.p.p. response times, suggesting that the difference in conduction velocities found in adults is already present at an early age. The latencies of inputs to muscle fibers were used to identify axon types and infer muscle fiber types in our study of polyinnervation by fiber type. We found that, contrary to predictions based on either twitch or tetanic tension, fast and slow muscle fibers are polyinnervated to the same extent.

**Methods**

*Motor unit tension recording*

Pregnant New Zealand White rabbits were obtained from ABC rabbitry (Pomona). Three age groups were included in this study: early (4-5 postnatal days), intermediate (8-9 postnatal days) and late (13-16 postnatal days).

Animals were anesthetized with inhaled Metofane followed by ether. The caudal portion of the trunk and the left leg were removed and placed in chilled Ringer's solution (150mM Na, 5mM K, 1mM Mg, 5mM Ca, 167mM Cl, 16mM glucose, and 14mM HEPES buffer, at pH 7.4) perfused with oxygen. The soleus muscle including all of its innervation to spinal roots L7, S1, and S2 were dissected free and placed in a chamber for electrophysiological recording. This chamber was maintained at $19.0 \pm 0.2^{o}C$ and continuously superperfused with oxygenated Ringer's solution. The muscle was pinned at its proximal tendon. A piece of 6-0 silk thread was tied to the distal tendon; the other end of this thread was attached to a piezoresistive tension gauge (Aksjeselskapet MikroElectronikk, Horten, Norway, Model AE 875).

A bipolar stimulating electrode was placed around the muscle, and the length of the muscle was adjusted so that stimulation via this bipolar electrode yielded the maximum twitch response and was consistent over the range of 40-60V. The maximum tension was tested periodically throughout each

recording session. The experiment was discontinued when the maximum tension declined below 80% of its initial value.

Ventral roots were divided into small filaments using jeweler's forceps. To prepare filaments containing single motor units, we varied the stimulus strength to ensure that only one component was present in the twitch response. Once single motor unit filaments were isolated, we stimulated them with a single pulse to elicit twitch responses and with trains of pulses (40 Hz, 600 ms) to elicit maximal tetanic responses. Both twitch and tetanus responses were digitized and stored on an IBM PC/XT for further analysis.

Motor units were identified as fast or slow contractile type on the basis of the rise time to peak of their twitch tension. At 19°C, motor units with twitch rise times of 250 ms or less were considered fast, while those with twitch rise times of 300 ms or greater were considered slow. These distinctions were based on the bimodal distributions of twitch rise times previously identified (Gordon and Van Essen, 1985).

Following measurements of single motor unit twitch and tetanic tensions, in some cases we measured the maximum tetanic tension of the whole muscle. Trains of pulses were delivered through the bipolar electrode to elicit maximal tetanic contraction of the muscle. This measurement was made at the end of a recording session because it sometimes caused extensive fatigue of muscle fibers.

*Intracellular endplate potential recording*

In one set of experiments, we isolated single motor units and identified their contractile types based on fast (<250 ms) or slow (>300 ms) twitch rise times. Motor units with intermediate rise times (250 - 300 ms) were not used. Fast and slow motor axons were drawn into separate suction electrodes. In most cases, we used one type of axon from a single ventral root, and the other type from a different ventral root; the other axons were cut away.

In a second set of experiments, we attempted to keep all of the innervation intact in order to measure the number of inputs per muscle fiber. To improve the resloution in counting inputs, the ventral roots contributing to soleus innervation were divided into 4 to 6 total filaments which elicited roughly comparable tension. The tendons were cut away from the muscle to reduce contractions during intracellular recording from muscle fibers (Barstad, 1962). The muscle was pinned as flat as possible, and connective tissue was removed from the surface. Recordings were made in two endplate-rich regions, one dorsal and one ventral to the main trunk of the soleus nerve within the muscle. Pulled glass microelectrodes were filled with 3M KCl and had impedences of 5-30 MΩ. Only muscle fibers on the surface were studied, because muscle fibers can become artifactually coupled to one other when the recording microelectrode is pushed through multiple laters of fibers (Soha et al., 1987).

E.p.p.'s were recorded following separate stimulation of each suction electrode. Throughout the experiment, we periodically checked to ensure that the filaments were securely within the suction electrodes. Graded stimulation of ventral root filaments was used to reveal multiple components to the endplate potential. When two inputs to a muscle fiber originated from the same stimulating electrode, the first-recruited component of the e.p.p. was subtracted (point-by point) from the total e.p.p. to obtain the trace of the second e.p.p.. The total number of inputs to a muscle fiber was the sum of inputs from each of the suction electrodes.

E.p.p.'s were amplified and stored on an IBM XT for on-line and off-line analysis. Signals were averaged over at least 3 traces, and the amplitudes and latencies of averaged e.p.p.'s were recorded. The latency, determined by computer, was defined as the time from stimulus onset to the time of the first voltage deflection above a small voltage threshold (0.25 - 0.3 mV). The nerve length was measured for each root from the proximal end of the dorsal roots to the nerve entry point into the muscle. Latencies were normalized by dividing by the length of the nerve from the stimulated ventral root.

## Results

*Twitch tension*

We measured twitch tension from 122 motor units in the early age group and 82 in the late age group. These motor units had a similar distribution of twitch tensions (as a percent of the maximum) as those previously described by Callaway et al. (1989). In the early age group, the mean twitch tension for the fast units was 5.7 $\pm$ 2.2% (s. d.) and the mean twitch tension for the slow units was 3.8 $\pm$ 1.9% (Figure 1a). As expected, these values decline with age; at the late age the means for fast and slow units were 2.6 $\pm$ 1.5% and 1.2 $\pm$ .74%, respectively (Figure 1b).

An estimate of the degree of initial polyinnervation at muscle fibers can be obtained from the ratio of mean motor unit tension (as a percentage of the maximum twitch tension for whole muscle stimulation) at the early age to the mean at the late age. Estimates for fast and slow motor units can be obtained by taking these ratios separately for the two types of motor units. Previous studies (Gordon, 1983; Callaway et al., 1989) based their estimates on twitch tension measurements. Their findings suggest that slow muscle fibers receive more inputs than fast muscle fibers. Our twitch tension data are in agreement with these estimates and suggest that, at the early age, fast muscle fibers receive 2.2 inputs while slow muscle fibers receive 3.1 inputs.

Figure 1. Twitch rise times of motor units plotted against motor unit size, expressed as a percent of the maximum twitch tension of the muscle. The twitch tensions of motor units used in this study yielded the expected bimodal distribution of twitch rise times at both early and late ages. At $19^{o}C$ , fast units have rise times $\leq$ 250 ms, while slow units have rise times $\geq$ 300 ms. At the early age, the mean motor unit size ($\pm$ s.e.m.) is 5.17 $\pm$ 0.28% for fast motor units (f) and 3.76 $\pm$ 0.24% for slow motor units (s). At the late age, the mean motor unit size is 2.6 $\pm$ 0.26% for fast motor units and 1.2 $\pm$ 0.11% for slow motor units.

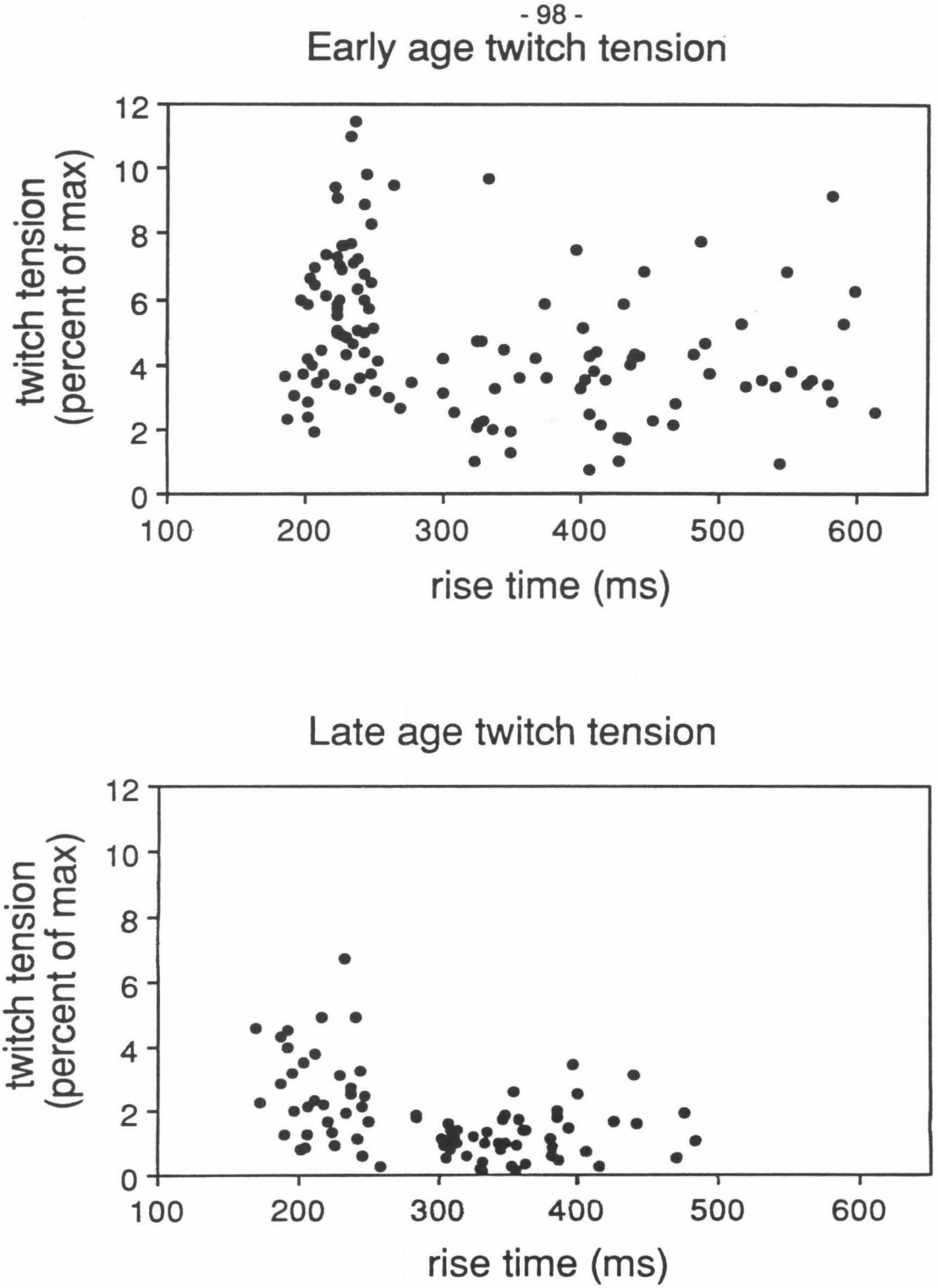

Early age twitch tension
twitch tension (percent of max)
0
2
4
6
8
10
12
100
200
300
400
500
600
rise time (ms)
Late age twitch tension
twitch tension (percent of max)
0
2
4
6
8
10
12
100
200
300
400
500
600
rise time (ms)

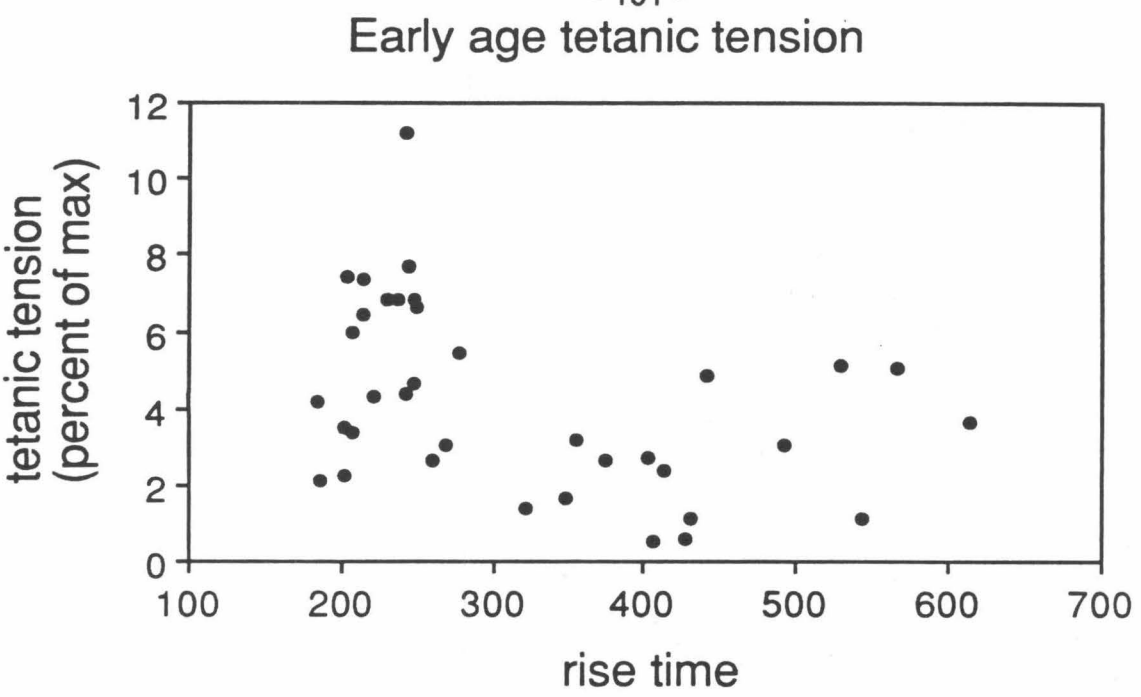
Figure 1

*Tetanic tension*

We measured tetanic tension of single motor units as a percentage of the maximum tetanic tension for the whole muscle for 32 motor units in the early age group and 42 motor units in the late age group. The mean tetanic tension at the early age was 5.6 $\pm$ 2.3% for fast units and 2.6 $\pm$ 1.6% for slow units (Figure 2a). At the late age, the mean tetanic tensions for fast and slow units were 2.5 $\pm$ 1.3% and 1.3 $\pm$ 2.3%, respectively (Figure 2b). Representative traces of twitch and tetanic tension measurements are shown in Figure 3.

We estimated the degree of polyinnervation based on tetanic tension measurements as a percentage of the maximum tetanic tension for whole muscle stimulation. Estimates using tetanic tension lead to the prediction that fast muscle fibers receive more inputs than slow muscle fibers. While the estimate for fast fibers of 2.3 inputs per muscle fiber is similar to the estimate we obtained using twitch tension measurements, the value for slow fibers is 1.3. This value is unlikely to be an accurate reflection of the actual degree of polyinnervation because several other studies indicate that at the early age all muscle fibers are polyinnnervated, and thus the estimate should be at least two inputs per fiber on average. Although estimates based on both twitch and

Figure 2. Twitch rise times plotted against tetanic tension (as a percentage of the maximum) of individual motor units at the early and late ages. A. The mean tetanic tension at the early age was 5.6±2.3% for fast motor units and 2.6 ± 1.6% for slow motor units. B. At the late age, the mean tetanic tension was 2.5 ± 1.3% for fast motor units and 1.3 ± 2.3% for slow motor units.


Early age tetanic tension
tetanic tension
(percent of max)
0
2
4
6
8
10
12
100
200
300
400
500
600
700
rise time

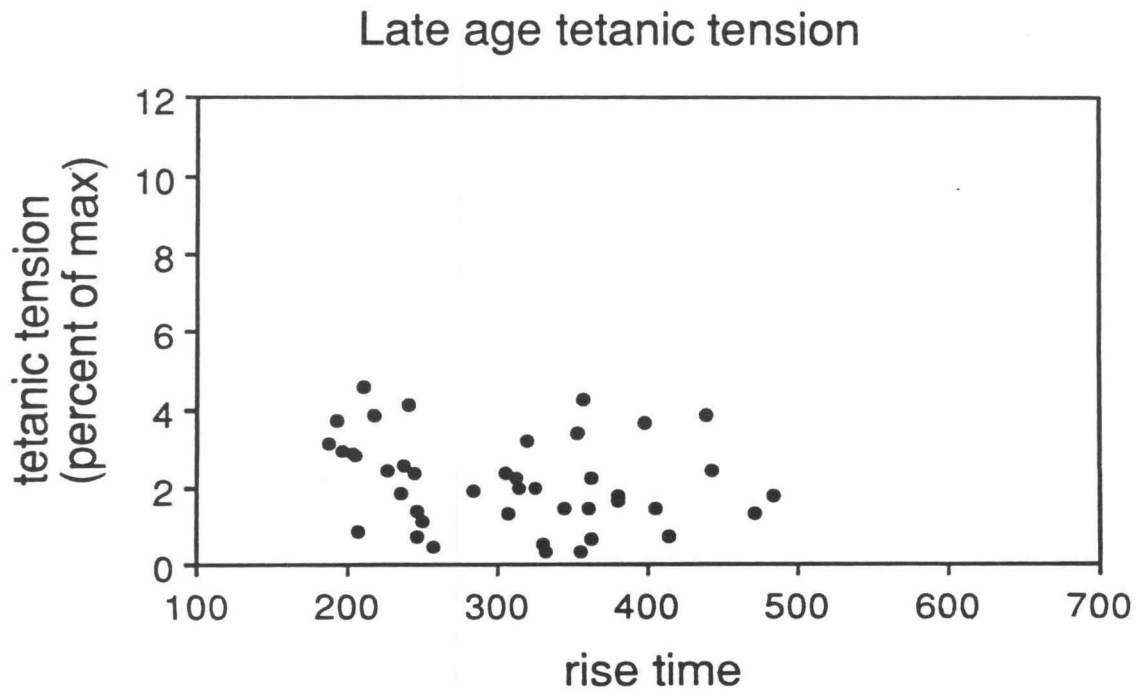

Late age tetanic tension
tetanic tension
(percent of max)
0
2
4
6
8
10
12
100
200
300
400
500
600
700
rise time

Figure 2

Figure 3. Examples of twitch and tetanic tension recordings used in this study. Panel A shows recordings from a fast motor unit in the late age group. The TTR for this unit is 0.23. Panel B shows recordings from a slow motor unit from the same rabbit as A. The TTR for this unit is 0.18.

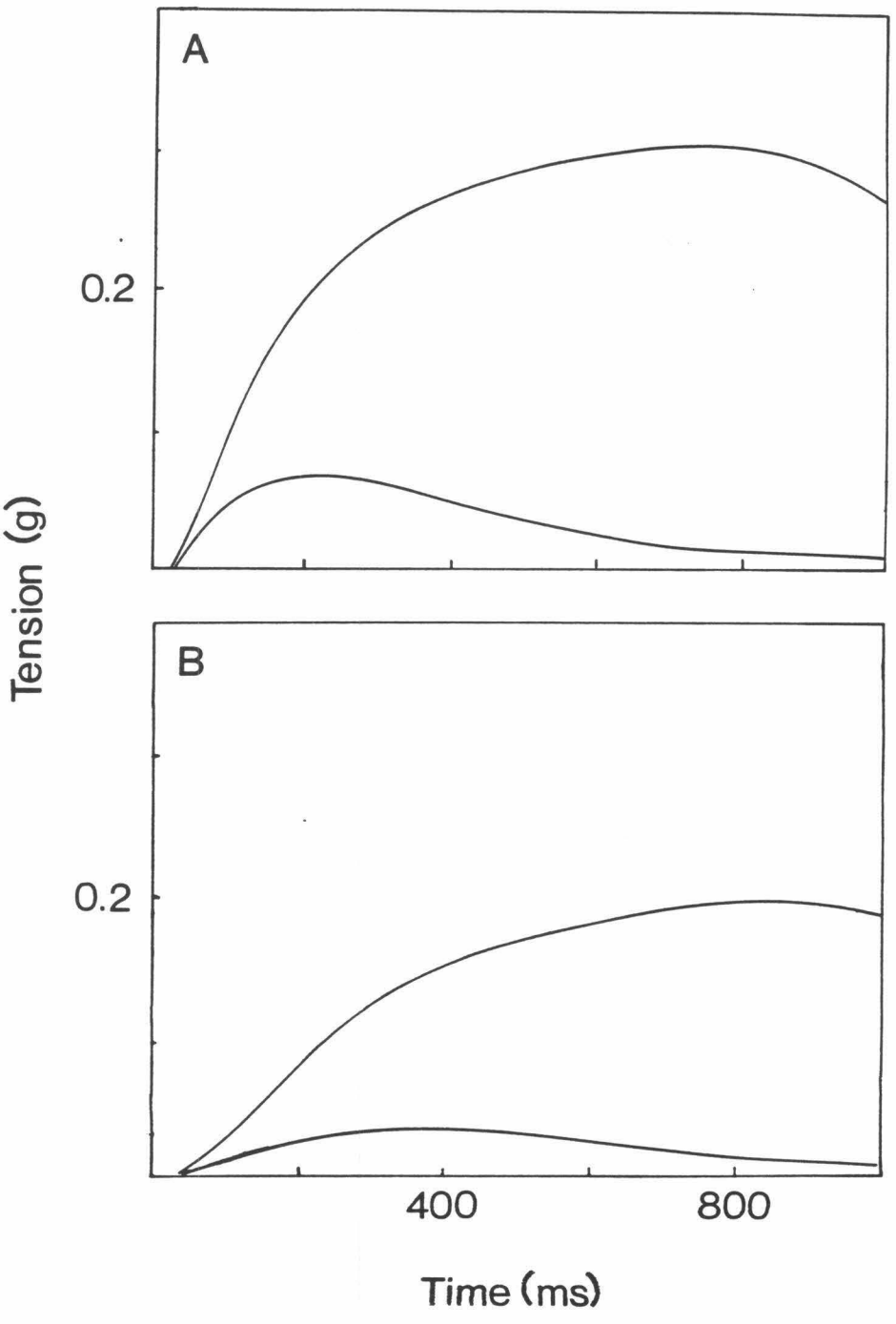



Figure 3

tetanic tension suggest a difference between fast and slow muscle fibers, the error in the measurement is substantial and these differences are not significant.

*Twitch/tetanus ratios*

For all three age groups, we obtained measurements of the twitch/tetanus ratios of single motor units by dividing the twitch tension (in grams) by the tetanic tension (in grams). The data are shown in Figure 4. At the early age, the mean twitch/tetanus ratio for the fast units was $0.24 \pm 0.01$ (s.e.m.); the mean for the slow units was $0.29 \pm 0.01$. At this age, slow motor units have significantly higher twitch/tetanus ratios than fast motor units ($p < 0.01$, student's t-test). In the middle age group, the mean twitch/tetanus ratios for fast and slow motor units were $0.27 \pm 0.02$ and $0.28 \pm 0.02$, respectively. These values did not differ significantly between fast and slow motor units ($p > 0.5$). In the late age group, mean twitch/tetanus ratios were $0.35 \pm 0.02$ for fast motor units and $0.21 \pm 0.01$ for slow motor units. Contrary to what we found in the early age group, at the late age group the twitch/tetanus ratios are higher for fast motor units than for slow motor units ($p < 0.001$). During the first two postnatal weeks, the TTR increases significantly for fast motor *units* ($p < 0.001$) and decreases significantly for slow *motor units* ($p < 0.001$). These results are summarized in Figure 5.

*Measurement of the degree of polyinnervation*

Because twitch and tetanic tension estimates on the degree of polyinnervation for fast versus slow muscle fibers are in conflict with one another, it is important to resolve the question using more direct methods. We used intracellular recording of endplate potentials because it allowed us to count the number of inputs directly. Furthermore, the degree of polyinnervation could be compared between fast and slow muscle fibers because, as the next part of this study shows, the latencies of the responses are indicative of axon type. We could then infer the muscle fiber type from the axon type(s) making synapses with a given muscle fiber. Thus, intracellular recording of e.p.p.'s gives information about both the number of inputs and their type, and thus makes it possible to compare the number of inputs to fast versus slow muscle fibers.

*Identification of fast and slow motor axons*

Filaments containing excusively fast or slow soleus axons were isolated from the ventral roots based on their twitch rise times to peak (see Methods). Each suction electrode contained 2 to 6 soleus axons, and these homogeneous filaments generated 9 to 20% of the total twitch tension of the muscle. We

Figure 4. A plot of twitch rise times against TTRs obtained from the early and late age groups. Mean TTRs (± s.e.m.) for the early age group were 0.29 ± 0.01 for slow motor units and 0.24 ± 0.01 for fast motor units. At this age, slow motor units have higher TTRs than fast motor units ($p < 0.01$, student's t-test). In the late age group, however, mean TTRs are higher for fast (0.35 ± 0.02) than for slow (0.21 ± 0.01) motor units ($p < 0.001$).

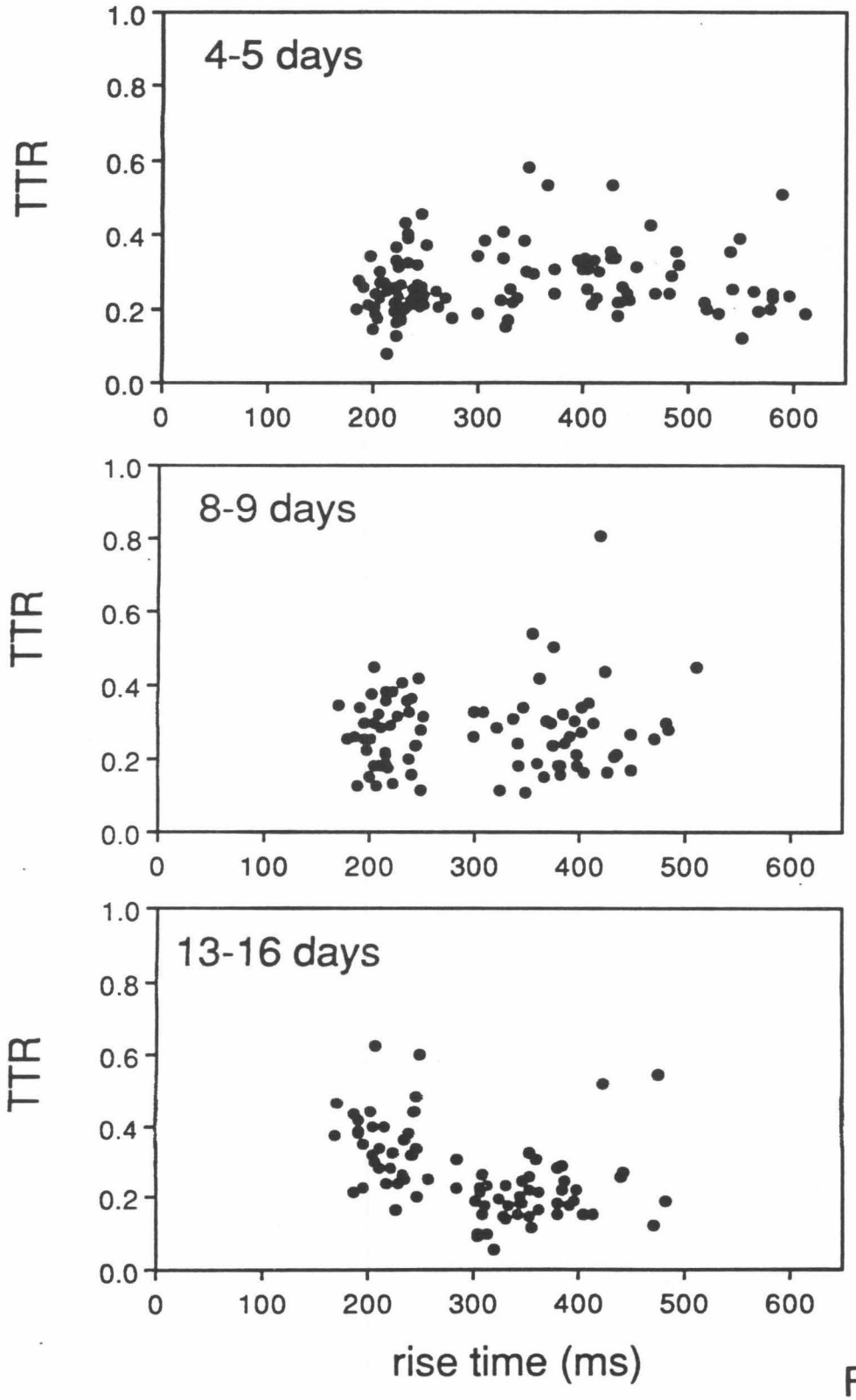

4-5 days
8-9 days
13-16 days
TTR
TTR
TTR
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
600
rise time (ms)

Figure 4

Figure 5. Histogram showing TTRs of fast and slow motor units at each age group. Error bars denote standard errors. During the first two postnatal weeks, slow motor units decrease their TTRs ($p < 0.001$) while fast motor units increase their TTRs ($p < 0.001$). In the intermediate age group, fast and slow motor units have about the same TTRs. Thus, during development, motor units change their TTRs, and these changes are different for fast and slow motor units.

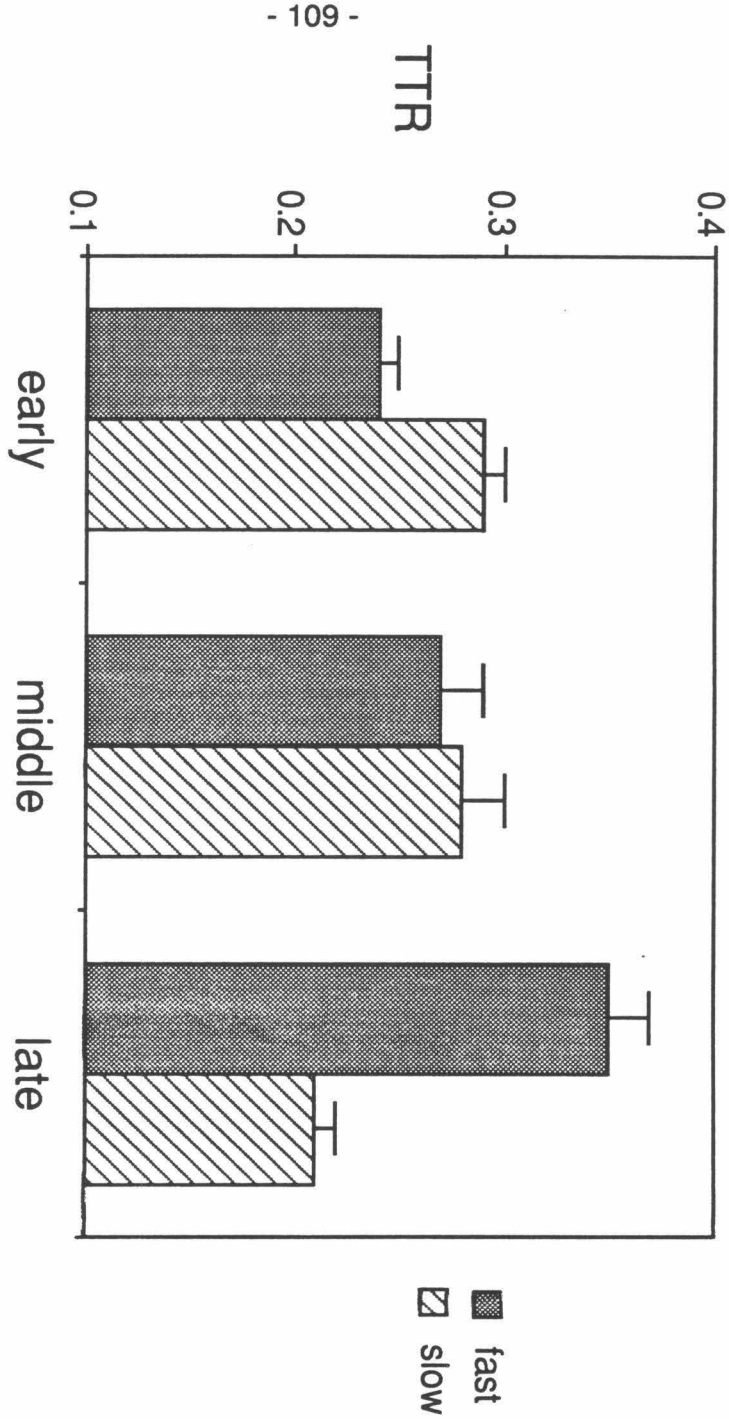



*Figure 5*

recorded latencies to e.p.p.'s following stimulation of fast and slow filaments. Fast and slow filaments usually came from different ventral roots, and because these ventral roots can differ in length by 2-3 mm, we normalized the latencies by dividing the latency by the nerve length. Thus the normalized latency is a measure of response time, which can be compared between axons from ventral roots of different lengths.

Five animals from the early age group and five animals from the intermediate age group had sufficient data to be included in this analysis, based on our requirement for at least 20 latencies recorded from each muscle. We recorded 20 to 35 e.p.p.'s from each muscle The normalized latencies (see Methods) were plotted for each muscle; histograms for all cases in the early and intermediate age groups are shown in Figures 6 and 7. In each case the mean normalized latency was greater for slow filaments than for fast filaments ($p < 0.001$, student's t-test, in 9 out of 10 cases; $p < 0.05$ in all cases). The average ratio of mean normalized latencies for slow:fast axons was 1.2.

Although the means were different, there was significant overlap between the distributions for fast and slow axons, similar to that reported in distributions of conduction velocities of fast and slow axons in adults (Bagust, 1974). The extreme upper and lower tails of the joint distribution, however, each containing 20% of the range, always contained only normalized latencies from slow and fast inputs, respectively.

Figure 6. Histograms of normalized latencies for fast and slow axons in 4-5 day old rabbits. The means for each group are indicated by arrows. In all cases, the mean normalized latency was higher for slow axons than for fast axons.

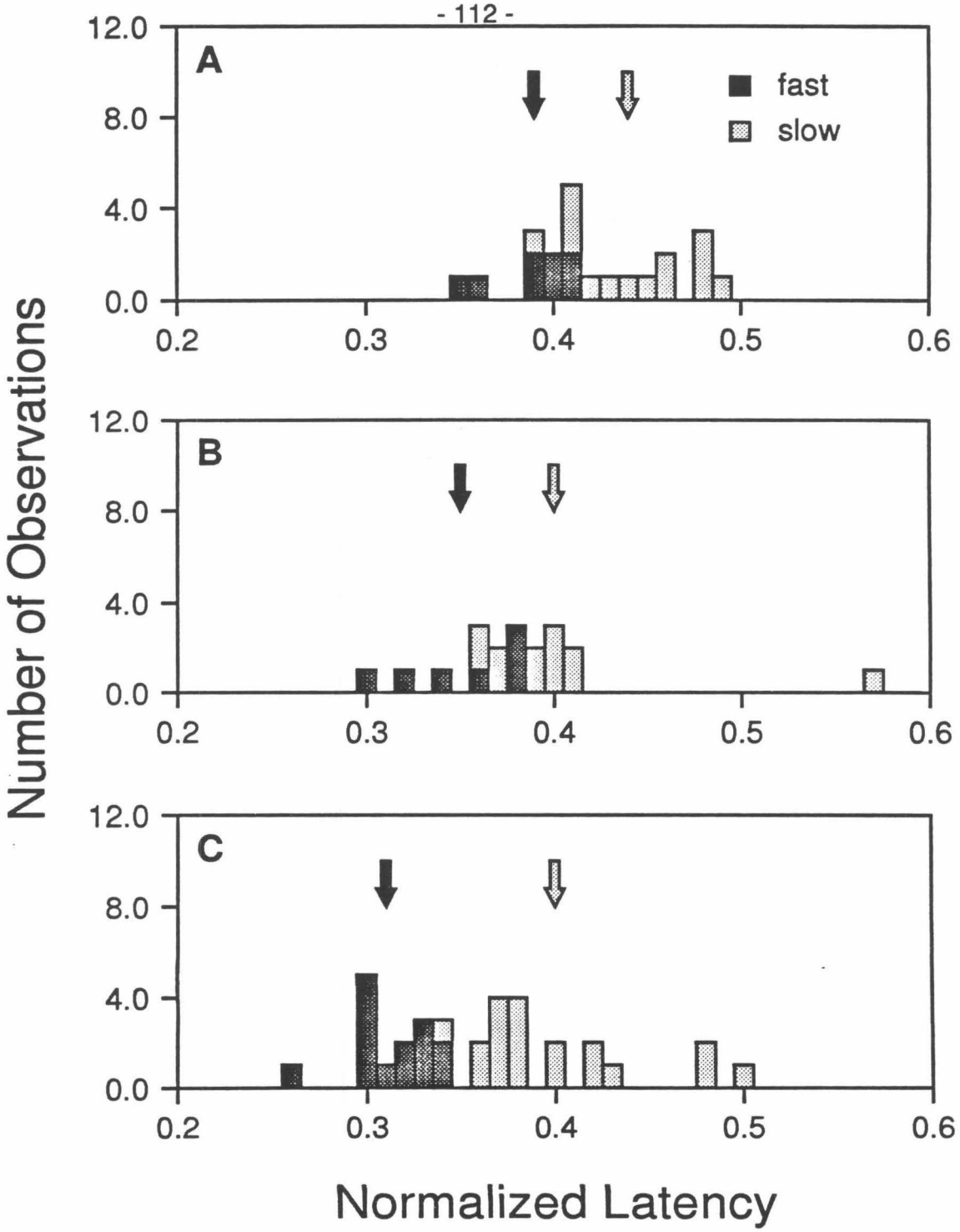

A
B
C
fast
slow
12.0
8.0
4.0
0.0
0.2
0.3
0.4
0.5
0.6
Number of Observations
Normalized Latency

Figure 6

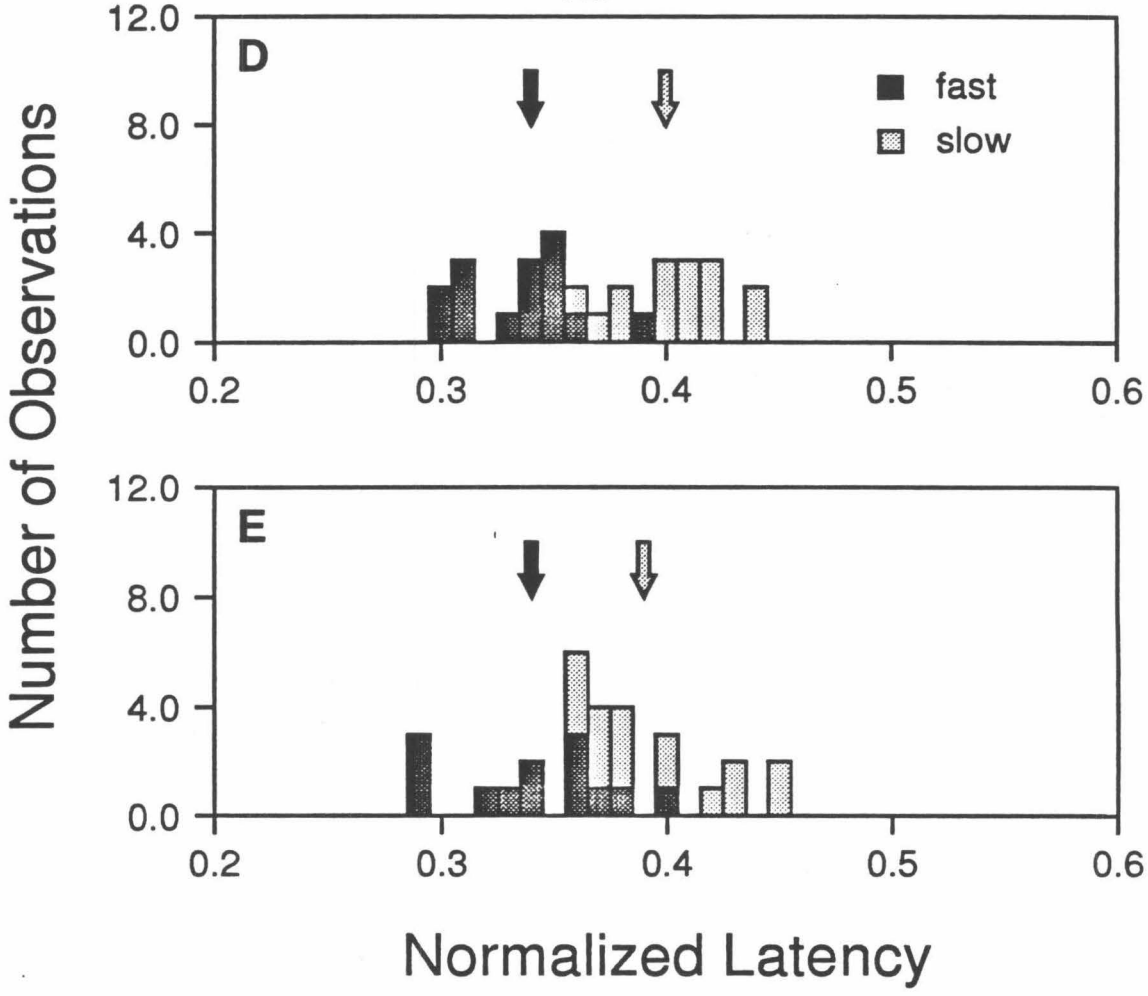

D
E
fast
slow
12.0
8.0
4.0
0.0
0.2
0.3
0.4
0.5
0.6
Number of Observations
Normalized Latency

Figure 7. Histograms of normalized latencies for fast and slow axons in 8-9 day old rabbits. As in Figure 6, the arrows indicate means for each group. Again, the mean normalized latency was higher for slow axons than for fast axons.

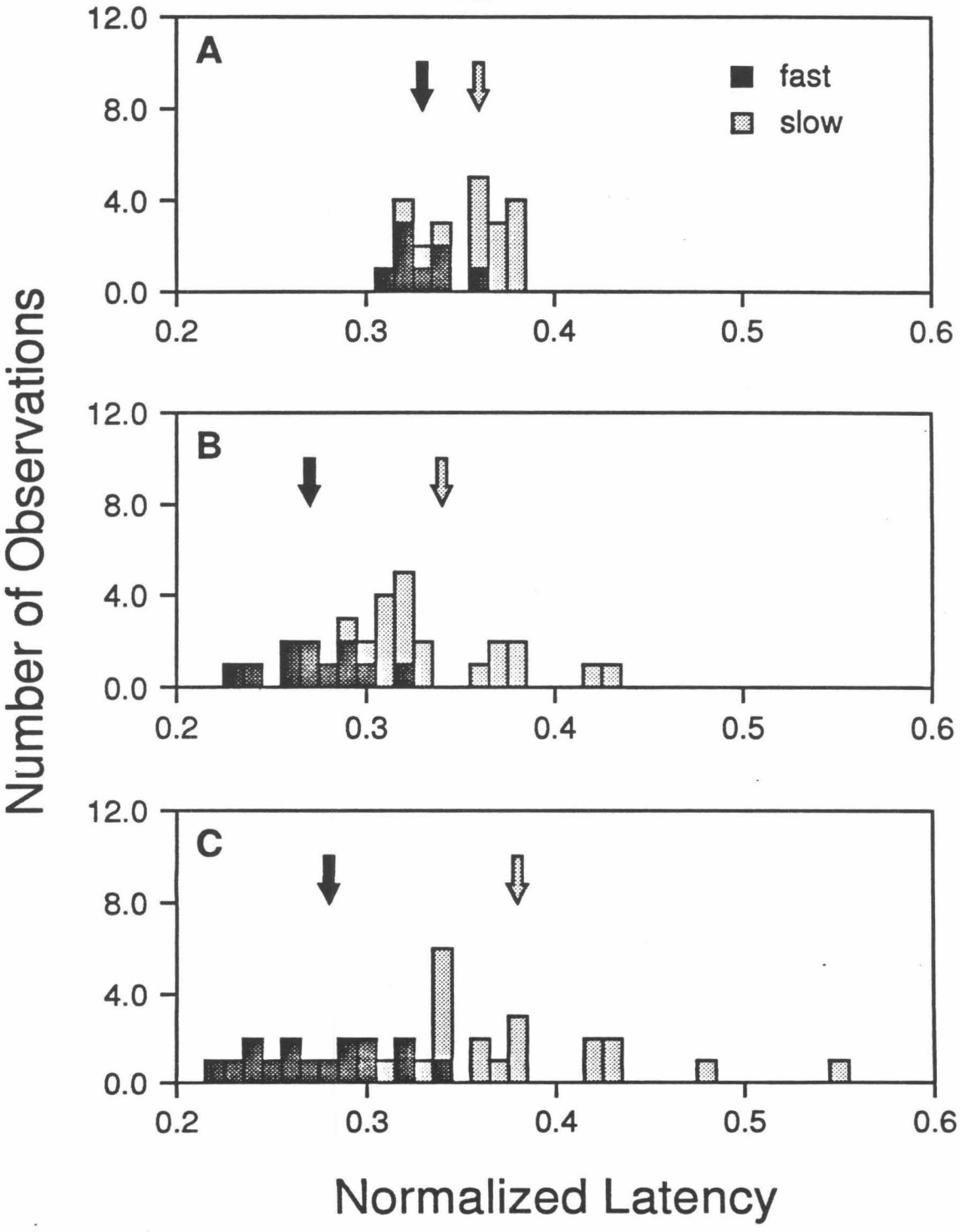
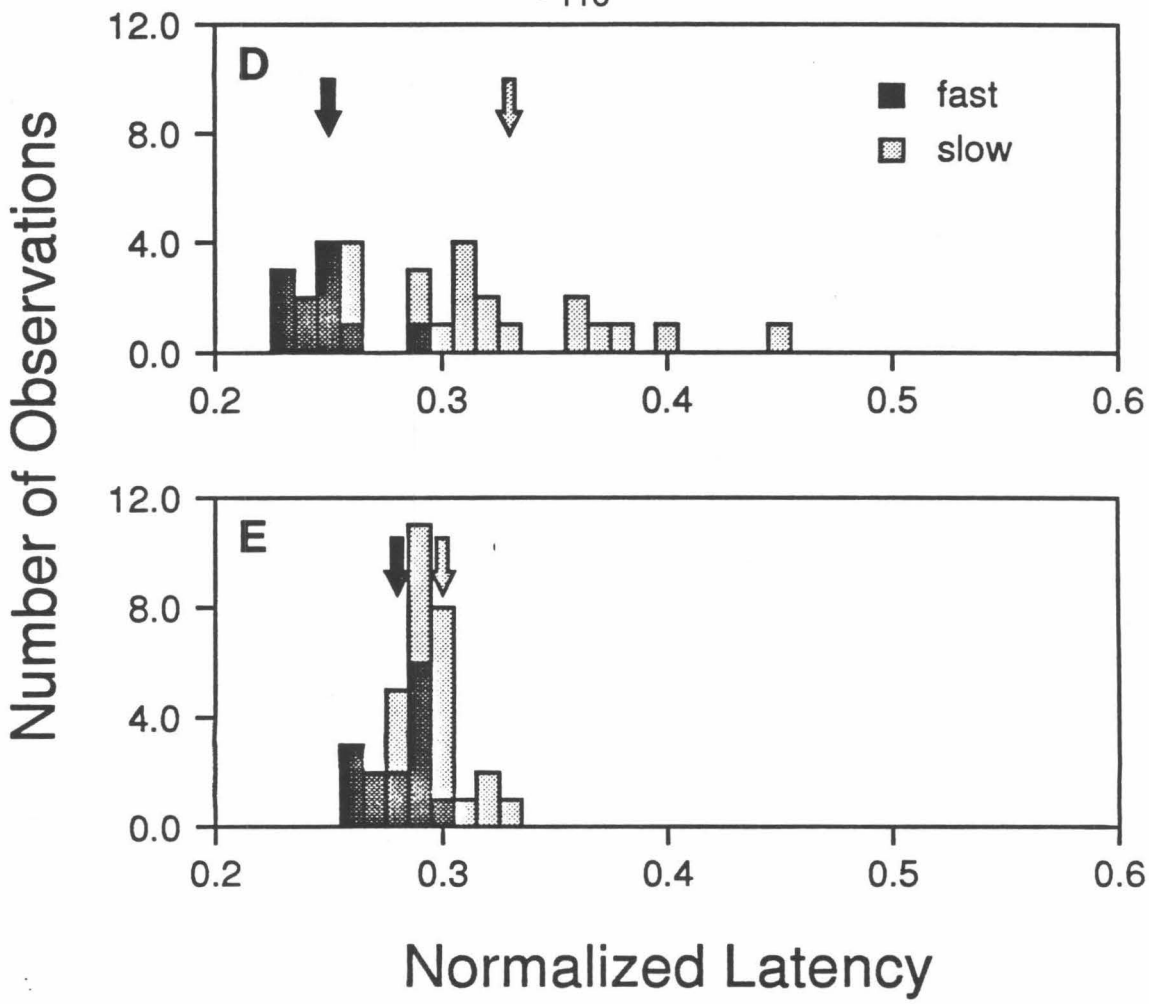

A
B
C
fast
slow
12.0
8.0
4.0
0.0
0.2
0.3
0.4
0.5
0.6
Number of Observations
Normalized Latency

Figure 7


D
E
fast
slow
12.0
8.0
4.0
0.0
0.2
0.3
0.4
0.5
0.6
Number of Observations
Normalized Latency

In order to increase the sample of fast and slow fibers available for analysis, we adopted strict identification criteria. The lower third of the normalized latency range was identified as fast. By this criterion, 14% of the fast axons in Figures 6 and 7 would be incorrectly identified. Because the normalized latency distributions of slow axons were less clustered, the upper half of the range was identified as slow. By this criterion, only 4.3% of the slow axons in Figures 6 and 7 would be incorrectly identified.

*Identification of muscle fiber types*

In separate experiments, intracellular e.p.p. recordings were obtained from muscles in which all the innervation was retained and was divided into a few large filaments to facilitate counting of inputs to individual muscle fibers (Fladby, 1987). Six animals from the early age group and three animals from the intermediate age group were included in these experiments. For each preparation, a normalized latency histogram was constructed. As discussed above, inputs whose normalized latencies were in the lower third of the distribution were highly likely to be fast, while those in the upper half of the distribution were highly likely to be slow. For each muscle fiber, each input was accordingly classified as fast, slow, or undetermined. When the majority of inputs to a muscle fiber were classified as the same type, the muscle fiber was classified as that type. Otherwise, the fiber was considered unclassified.

Because inputs whose normalized latencies were in the center of the distribution could not be identified unambiguously, there were many muscle fibers for which a majority of inputs were unknown, and thus these fibers remained unidentified. In the early age group, the number of inputs was determined for 127 muscle fibers; of these, 53 were classified as fast and 10 were classified as slow. In the intermediate age group, 29 slow and 25 fast fibers were identified out of 97 fibers for which the number of inputs was determined. This method of identifying muscle fibers was conservative and had a low error rate. For example, if the mean number of inputs to muscle fibers is three, then two inputs must be identified incorrectly for the muscle fiber to be identified incorrectly. At an error rate of 10% for each input, muscle fibers would be identified incorrectly only 1% of the time.

*Degree of polyinnervation*

We assessed the degree of polyinnervation for at least 20 muscle fibers in each muscle. The overall mean degree of polyinnervation at the early age was 2.6 $\pm$ 0.1 (s.e.m.) inputs per muscle fiber; at the intermediate age this figure decreased to 1.6 $\pm$ 0.1 inputs per fiber. These values are in agreement with those obtained from twitch tension estimates and are higher than those obtained using tetanic tension estimates. We found 6 singly innervated muscle fibers at the early age group, or 4.7% of the total fibers, in contrast with

previous observations that all muscle fibers are polyinnervated in the neonatal rabbit soleus (Brown et al., 1976). These apparently singly innervated fibers may be indicative of a modest degree of axon damage that may have been sustained when ventral roots were separated into filaments. However, they may in fact reflect early appearance of some single innervation. At the intermediate age group, we found that about half of the muscle fibers were singly innervated, consistent with previous findings (Soha et al., 1987).

The degree of polyinnervation for fast versus slow fibers was assessed from measurements on muscle fibers whose type was identified. At the early age, the mean number of inputs at slow muscle fibers was $2.6 \pm 0.2$ (s.e.m.); the mean at fast muscle fibers was $2.6 \pm 0.1$. The percentages of muscle fibers with different degrees of polyinnervation are shown in Figure 8. These means were not significantly different from each other ($p > 0.9$, student's t-test). Furthermore, neither group was different from the overall mean for the age group ($p = 0.86$), suggesting that there was no bias introduced in classifying only the subset of muscle fibers in which a majority of inputs fell in the tail of the normalized latency distribution. Thus, using this method for counting inputs at individual muscle fibers, we find that fast and slow muscle fibers are polyinnervated to the same extent at the early age, contrary to the predictions from either twitch or tetanic tension measurements (Table 1).

At the intermediate age, the mean number of inputs to slow muscle fibers was $1.5 \pm 0.1$, and the mean for fast muscle fibers was $1.3 \pm 0.1$. Again, the degree of polyinnervation was not different between fast versus slow muscle fibers ($p = 0.2$). In this case the means may have been less than the overall mean from the age group ($p = 0.1$). Such a difference may reflect a bias for identifying singly innervated fibers. In these fibers, only one input must be identified (fall in the lower third or upper half of the normalized latency distribution). At this age, about half of the muscle fibers are singly innervated. Although our data may have been biased toward fewer mean inputs per fiber, the similarity in the degree of polyinnervation between fast and slow muscle fibers at this age is consistent with that found in previous studies (Soha et al., 1987)

The small number of slow muscle fibers obtained in the early age group in this study may in part be explained because the soleus has a ratio of slow:fast muscle fibers of about 3:7 (Gordon, 1983). Some of the distributions in this data set were skewed toward the low end, with relatively few normalized latencies in the upper half of the range. It did not seem reasonable to relax our criteria for identifying slow axons because the error, from distributions of known axon types, would become rather high.

*Specificity in innervation of fast and slow muscle fibers*

Using information about normalized latencies, it was possible to estimate the frequency at which fast and slow axons occur together at a single endplate. In this analysis, all muscle fibers were included, even those whose identity was not determined because the majority of inputs were unidentified. For this reason, we were more conservative in our criteria for identifying axon types, and we used only the upper and lower thirds of the distributions. In the early age group, 6 muscle fibers out of 127, or 4.7%, had both fast and slow inputs. In 2 cases, these inputs were in the extreme 20% of the distribution, where there were no errors in the data set of physiologically identified motor axons (Figure 6). Furthermore, in the experiments in which fast and slow filaments were stimulated separately, one muscle fiber in the early age group, or 0.75% of all the fibers in that part of the study, was innervated by both a fast and a slow axon. In the intermediate age group, there were no cases in which muscle fibers were demonstrably polyinnervated by both a fast and a slow motor axon.

Figure 8. Histograms of the percent of fibers at each degree of polyinnervation for fast and slow axons. The mean degree of polyinnervation did not differ between fast and slow muscle fibers.

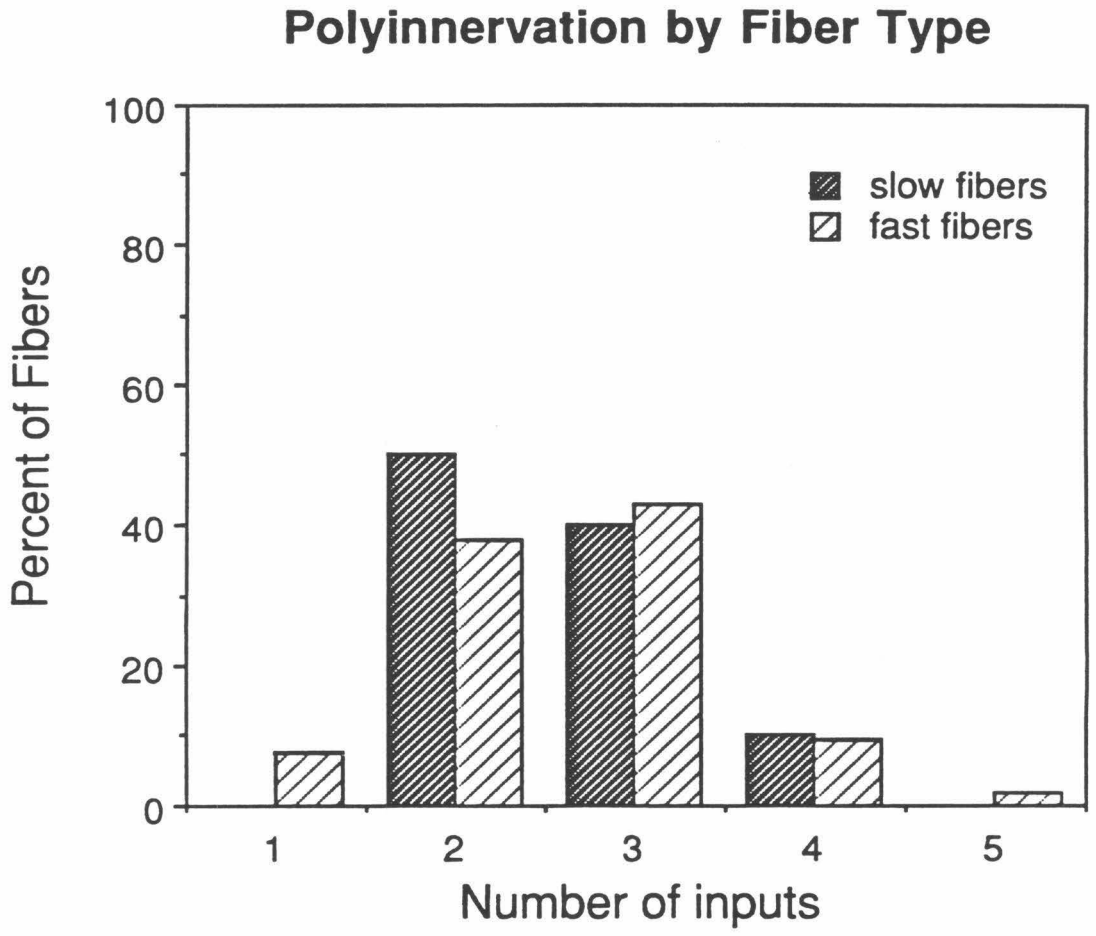

Polyinnervation by Fiber Type
100
80
60
40
20
0
Percent of Fibers
slow fibers
fast fibers
1
2
3
4
5
Number of inputs

Figure 8

Table 1. Estimates of the number of synapses per muscle fiber based on twitch and tetanic tension measurements and on intracellular recording.

**Table 1. Estimated degree of polyinnervation**

| Method | Fast Muscle Fibers | Slow Muscle Fibers |
|---|---|---|
| Twitch tension (Callaway et al.) | 2.1 | 3.4 |
| Twitch tension, this study | 2.2 | 3.1 |
| Tetanic tension | 2.3 | 1.1 |
| Intracellular recording | 2.6 | 2.6 |

## Discussion

*Twitch/tetanus ratios*

The observed changes in the distribution of twitch/tetanus ratios occur at a time during postnatal development when major changes in myosin isoforms take place in muscle (Hoh and Yeoh, 1979; Whalen et al., 1981; Butler-Brown and Whalen, 1984). In the rat soleus, muscle fibers contain embryonic and neonatal forms of myosin at one week of age. These isozymes are largely replaced by adult isoforms after about two postnatal weeks. The changes in isozymes of contractile proteins are thus likely to contribute to changes in physiologically observed twitch/tetanus ratios, and may influence differences between fast and slow motor unit contractile properties.

In addition to changes in mysosin isozymes, muscles undergo changes in other types of proteins during this period of postnatal development. For example, muscle sodium channel transcripts, which are expressed to different extents in fast and slow muscles (Trimmer et al., 1990), are developmentally regulated in postnatal rats. However, it is not known how this change is reflected in individual muscle fibers.

*Degree of polyinnervation*

Estimates of the degree of polyinnervation based on motor unit tensions are indirect, and are complicated by the fact that tension is the product of the number of muscle fibers in the unit, the cross-sectional area of each fiber, and the tension per unit area (the specific tension). The cross-sectional area of muscle fibers increases to the same extent in both fast and slow muscle fibers during synapse elimination (Gordon, 1983) However, little is known about the relative changes in specific tension. Estimates of the degree of polyinnervation assume that specific tension changes to the same extent in all muscle fibers. Because we now have an independent measure of the degree of polyinnervation, these estimates suggest that specific twitch tension increases relatively more in fast muscle fibers than in slow muscle fibers, while specific tetanic tension increases relatively more in slow muscle fibers. These changes in specific tension would be consistent with the changes in the twitch/tetanus ratio, which is also a measure of the ratio of specific twitch tension to specific tetanic tension. This view of specific tension accounts for the discrepancies between estimates of the degree of polyinnervation based on twitch versus tetanic tension, and reconciles them with results from intracellular recording. These differences in specific tension could be examined directly using glycogen depletion of the muscle fibers in a motor unit of known contractile type and force, after which tension per unit area could be compared directly between fast

and slow muscle fibers.

Our measurements on the degree of polyinnervation required a method for identifying muscle fiber type. In a separate set of experiments, we established that e.p.p. latencies could be used as a relatively reliable indicator of fiber type. Because conduction velocities are different for fast and slow motor axons in adults (Bagust, 1974; Wuerker et al., 1965), we predicted that we would find differences in e.p.p. latencies. Within an animal, it is likely that the differences in normalized latency are due to differences in axonal conduction velocity. We normalized latency by dividing by nerve length to avoid introducing systematic differences when fast and slow axons came from ventral roots of different lengths. It is important to note, however, that the normalized latency, although it has units of seconds per meter, is not simply the inverse of axonal conduction velocity. The nerve length includes only the extramuscular portion of the nerve. The latency includes conduction time within the muscle as well as synaptic delays in addition to conduction time along the measured part of the nerve. Although the intramuscular part of the nerve only constitutes a small part of the total nerve length, it may contribute significantly to the measured latency. Within the muscle, nerve branches may conduct more slowly because of their small size and because myelination is incomplete during the first two postnatal weeks (Bixby, 1981).

These findings suggest that muscle fiber types can be identified using e.p.p. response latenties. Using this method, we find that fast and slow muscle fibers are polyinnervated to the same extent at the early age. Because the number of inputs remains the same for fast and slow muscle fibers at the intermediate age, we can infer that fast and slow muscle fibers eliminate synapses at the same rate during this time interval.

The finding that fast and slow axons have different latencies, probably indicative of differences in conduction velocity, further reflects that fast and slow motor units are functionally distinct and that some of their distinguishing properties are evident early in postnatal development. However, while the mean latencies are different between fast and slow axons, the two groups are not sharply divided in the way that many properties of fast and slow muscle fibers are.

*Specificity of connections*

In intracellular recording experiments which included all the innervaiton in 4-5 day rabbits, 4.7% of all the muscle fibers received at least one fast and one slow input, where input types were assigned in the lower or upper third of the normalized latency distributions. One way to assess the degree of specificity in the present material is to consider the observed groupings of identified fast and slow inputs and compare their distributions to those predicted

for random innervation. The probability that a given identified input is fast (P(F)) or slow (P(S)) is the fraction of the total identified inputs that is fast or slow, respectively. In our experiments, P(F) = 0.88 and P(S) = 0.12. For perfect specificity, we would expect no instances where fast and slow identified inputs innervate a given endplate. For random innervation, the probability of finding a given combination of inputs can be computed from the binomial distribution. When there are two identified inputs,

$$P(\text{different types}) = 2P(F)P(S) = 0.21$$
$$P(\text{same type}) = P(F)^2 + P(S)^2 = 0.79$$

where P(different types) represents the sum of the probabilities of each combination in which the identified inputs are different. Likewise, P(same type) represents the sum of the probabilities of each combination in which all the identified inputs at a given endplate are of the same type. The ratio of different types: same type for random innervation, $r_{random,}$ is 0.27. The observed ratio, $r_{actual}$, is 0.05 (n = 40). We define a specificity bias, B:

$$B = ( r_{random} - r_{actual}) / (r_{random})$$

Thus, for two identified inputs, the specificity bias is 0.81. When we consider cases in which there are three identified inputs,

$$P(\text{different types}) = 3P(F)^2P(S) + 3P(F)P(S)^2 = 0.32$$

$$P(\text{same types}) = P(F)^3 + P(S)^3 = 0.68$$

and $r_{random} = 0.47$. In this case, $r_{actual} = 0.33$ ($n = 16$). Thus, for three identified inputs, $B = 0.30$.

Because there are few cases in which greater than three inputs are identified ($n = 5$), a reasonable estimate of the degree of specificity cannot be obtained for those cases. We can use a weighted average of B to obtain an estimate of the overall degree of specificity:

$$B_{average} = (n_2B_2 + n_3B_3) / (n_2 + n_3)$$

where $n_i$ represents the number of cases with i identified inputs, and $B_i$ represents the specificity bias in cases of i identified inputs. From our data, $B_{average} = 0.66$. This represents a degree of specificity comparable to what has been found in the rat (Thompson et al., 1984) and mouse (Fladby and

Jansen, 1990) soleus muscles.

The average number of inputs (± s.e.m.) at endplates with two or three identified inputs, respectively, was 2.5 ± 0.1 and 3.3 ± 0.1. This degree of polyinnervation was significantly less ($p < 0.001$, student's t-test) at endplates with two identified inputs than at those with three identified inputs. Thus, there is a correlation between specificity and degree of polyinnervation; this correlation might be suggestive of selective synapse elimination.

The presence of some precision in connections between fast and slow motor neurons and the corresponding muscle fiber types at early ages is consistent with specificity in initial synapse formation. While the mechanisms that give rise to this specificity early in development are not known, the available evidence supports mechanisms involving differential timing of synapse formation (see Chapter 1), as well as chemospecific recognition of appropriate target muscle fibers (Soileau et al., 1987). Several studies agree that connections between motor neurons and muscle fibers are specific at birth, but lack the precision seen in adult muscles. These findings raise the issue of how precision in connections is improved during postnatal development.

One plausible mechanism involves respecification of muscle fiber types according to neuronal firing patterns. Muscle fiber types can be experimentally altered in response to electrical stimulation or cross-innervation by nerves of the opposite type (Bagust et al., 1974b; Salmons and Sreter, 1976; Jolesz and

Sreter, 1981; Gorza et al., 1988). It would be interesting to examine the extent to which this type of mechanism is important during normal maturation of motor unit types. Another possibility is that inappropriate connections are selectively removed during postnatal synapse elimination. This mechanism is likely to play a role in refining topography in projections to some muscles (Brown and Booth, 1983; Laskowski and High, 1989; see also Chapter 2). Moreover, at least some refinement in specificity coincides with the emergence of single innervation in rat and mouse soleus muscle (Thompson et al., 1984; Fladby and Jansen, 1990); the present study extends these results to rabbit soleus. Thus, it is feasible that synapse elimination sharpens specificity early in postnatal development.

In order to understand the relative importance of these mechanisms, it would be useful to generate computer models of the development of specific connections. Such models have been useful in understanding neuromuscular synapse elimination (Van Essen et al., 1990), as well as the development of retinotectal topography (Fraser and Perkel, 1990) because they allow several combinations of mechanisms to be tested. Computer models would assist in the generation of hypotheses which could be tested experimentally, and would thus be a useful tool in understanding the interactions between developmental mechanisms which give rise to specificity of connections.

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