POSTNATAL DEVELOPMENT OF MOTOR UNITS IN RABBIT AND RAT SOLEUS MUSCLES

Thesis by

Herman Gordon

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ABSTRACT

The development of motor unit properties in the soleus muscle of rabbits and rats was used to study the control of innervation and elimination of synapses from mammalian skeletal muscle fibers and the differentiation of those muscle fibers into various twitch types.

1. Motor unit twitches with distinctly different time courses were found in rabbit soleus muscle at a stage in development when all muscle fibers were polyinnervated. This observation implies that (1) muscle fibers have already begun their physiological differentiation into twitch types while still polyinnervated and (2) motor neurons of a specific type preferentially polyinnervate muscle fibers of a corresponding type.

2. It has recently been claimed that synapse elimination occurs preferentially among motor neurons from the more rostral of the two spinal roots contributing to the soleus muscle of the rat. Using an assay based on measurements of motor unit twitch tensions, it was found, contrary to the previous claim, that synapses were lost to the same extent by motor neurons passing through all contributing spinal roots to both the rabbit and rat soleus muscles.

3. Two lines of evidence indicate that rabbit soleus motor neurons redistribute their terminals at a time after wholesale polyinnervation has been lost from the muscle. (1) Between 11 and 18 days and 5 weeks of age, the frequency of histochemically defined type I fibers increases from 30% to 65% while the incidence of physiologically defined slow motor units does not obviously change. (2) Over the same time period, the ratio of average slow twitch tension to average fast twitch tension quadruples after correction for changes in muscle fiber cross sectional area. I hypothesize

that during this 3 week time window, slow twitch motor neurons take over end plates previously occupied by fast twitch motor terminals.

4. Activity has previously been shown to play a role in the overall rate of synapse elimination. I have conducted preliminary experiments to address whether a competition on active muscle fibers between terminals of active and tetrodotoxin-inactivated motor axons results in a preferential retention of active connections. With the paradigm used, there was at most a small bias favoring the survival of active synapses.

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GENERAL INTRODUCTION

The mammalian nervous system does not develop rigidly and inexorably from a genetic program. Instead, mammalian genes seem to lay out a basic plan with many decisions suspended to a remarkably late stage of development. Cells proliferate, differentiate, migrate, and form interconnections according to laws handed down at conception. But the expression of these laws depends in large measure on later interactions between cells and the organism's environment. These interactions lead to decisions which gradually pare down the wealth of possibilities inherent in the original genetic plan. Such a plastic and adaptable approach to development has several advantages. First, as a system becomes more and more complex, mistakes, such as the failure of a stem cell to divide at a given time, become ever more likely and take on potentially ever more catastrophic significance. The mammalian nervous system in the adult and especially in development avoids depending on the existence of unique cells and specific connections. Second, developmental flexibility has enabled evolutionary flexibility, making possible the evolution of highly sophisticated nervous systems over impressively short time spans.

The basis of mammalian neuronal plasticity lies in two fundamental programs for wiring and rewiring connections. The first involves whole cells. Early in development, motor neurons, as an example, are overproduced only to die around the time at which the first neuromuscular synapses are formed (Hamburger & Levi-Montalcini, 1949; Harris-Flanagan, 1969). The advantage of such a strategy in development remains an enigma, but it is thought that developmental cell death may help to regulate source and target pool sizes (Hamburger & Oppenheim, 1982). In vertebrate cell death it is not known whether individual cells are preprogrammed to die or to what extent their individual fates reflect interactions with other cells

and with the environment. The second program involves the regulation of synaptic connections between cells. A tractable class of such synaptic plasticity lies in a dramatic reorganization of connections which occurs during normal development. During prenatal development, connections between neuronal cells are massively overproduced, and later whole synapses and associated terminal processes are eliminated (Redfern, 1970; Purves & Lichtman, 1980; Grinnell & Herrera, 1981; Van Essen, 1982). Overproduction of connections followed by elimination of synapses allows for a wealth of final patterns of connectivity from a single genetic plan. It is toward the isolation of potential controlling factors on the phenomenon of synapse elimination and on the development of neuromuscular connections in general that the research in this thesis is addressed.

Synapse Elimination in Mammalian Muscle

Initial hyperinnervation followed by elimination of synapses appears to be a fact of life for many developing organs. It occurs in mammalian skeletal muscle (Redfern, 1970; Bagust, Lewis, & Westerman, 1973; Brown, Jansen, & Van Essen, 1976; Bixby & Van Essen, 1979a), visual cortex (Shatz & Stryker, 1978; LeVay, Stryker, & Shatz, 1978), cerebellum (Crepel, Mariani, & Delhaye-Bouchaud, 1976), autonomic ganglia (Lichtman, 1977), and projections to motor neurons in the spine (Ronnevi & Conradi, 1974; Ronnevi, 1979). Of these, skeletal muscle provides the most approachable system for detailed study. In muscle, the wholesale elimination of synapses is dramatic, fast, accessible, and potentially more simple. As many as 75% of the peak number of synapses are lost in a matter of two weeks (Brown et al., 1976). The source, target, and connective are well

segregated, and the targets bear only one synapse in the end. Furthermore, investigation is facilitated by the use of *in vitro* assays (Redfern, 1970; Brown et al., 1976).

Polyinnervation and synapse elimination in developing muscle were first discovered in the early part of this century with anatomical techniques (Tello, 1917; Boeke, 1932). The observation appears largely to have been forgotten until 1970 when Redfern reported physiological evidence for neuromuscular polyinnervation. Subthreshold end-plate-potentials in curarized neonatal rat diaphragm showed multiple components upon graded stimulation of the phrenic nerve. Redfern hypothesized that the multiple components indicated that multiple motor axons innervated the same muscle fiber and were recruited at distinct thresholds of nerve stimulation. Since he found that muscle fibers at 16 to 18 days after birth showed only single-component end-plate-potentials, there had to be a period in which the redundant synapses were suppressed or eliminated. Brown et al. (1976) then showed that the multiple inputs were confined to single end plates on each muscle fiber using a zinc-iodide stain for terminal processes and iontophoresis of acetylcholine to identify synaptic regions by desensitization.

The loss of neuromuscular polyinnervation in general represents a net loss of synapses. In the rabbit soleus muscle, for example, the numbers of muscle fibers and motor neurons are constant after birth (Bixby & Van Essen, 1979a; Bixby, Maunsell, & Van Essen, 1980), yet approximately 75% of the synapses are lost during the first two weeks after birth (Chapters 2 & 3). On the other hand, rat lumbrical muscles prove an exception to the rule. These muscles are polyinnervated at birth but at that time contain only one half of the adult number of muscle fibers (Betz,

Caldwell, & Ribchester, 1979). During the first 10 days after birth, myogenesis continues to occur, and synapses are formed on the new muscle fibers at a rate equal to their loss from older fibers. Rather than a net loss in the lumbrical muscle, there is instead a redistribution of synapses, of simultaneous sprouting to uninnervated muscle fibers and withdrawal from polyinnervated fibers. After 10 days of age, the remaining polyinnervation is removed for an eventual modest decline in the total number of synapses in the muscle. Nonetheless, even in this exceptional muscle, many of the muscle fibers and probably all of the motor neurons are involved at one time during their development in polyinnervation and synapse elimination.

Motor Units

The approach to neuromuscular synapse elimination taken in this thesis is to study the development of motor units. A motor unit consists of a motor axon and all the muscle fibers that it innervates. For Sherrington (1929), who coined the term, the motor unit provided the irreducible unit of motor control in the adult mammal. For developmental studies, the motor unit represents the fundamental unit of wiring and enables the investigator to follow changes in the pattern of that wiring. In particular, the size of a motor unit, the number of muscle fibers innervated by a single motor axon, serves as an index of the competitive success of a motor neuron. Because only one motor terminal eventually remains on a muscle fiber, there is necessarily a competition between motor neurons to retain connections to particular muscle fibers. What is the basis of this competition?

Motor unit sizes will decrease on average during the period of synapse elimination and may decrease differentially according to specific properties of the component motor neuron and muscle fibers. It is hoped that the pursuit of changes in motor unit size in relation to other motor unit properties may help elucidate the factors, if any, on which motor neurons compete to retain synapses during the loss of polyinnervation. For instance, specific motor neurons may be required for whatever reason to be connected to specific muscle fibers. Polyinnervation followed by a directed synapse elimination might then facilitate the achievement of a certain target specificity. Similarly, motor neurons may possess an intrinsic capacity to innervate specific numbers of muscle fibers and may require a period of plasticity during which they can be matched to an appropriate target pool size. The research strategy, then, is to study the development of motor unit properties as a way of assessing the salient influences behind the developmental changes in the pattern of neuromuscular wiring. The motor unit approach contrasts with studies from the perspective of the synapse or the muscle fiber which confront the question of <u>how</u> synapses are eliminated. The former approach deals with the question of <u>which</u> synapses are eliminated, from which one hopes to infer on what basis they are eliminated.

A Naive View

Mono-innervation in the adult allows for a segregation of motor control into discrete motor units. There is no cross-wiring, and the control network is relatively simple. Given a polyinnervated muscle, the need to eliminate confusing connections can be easily appreciated. But why

polyinnervate muscle in the first place? Perhaps it is all part of a grand developmental strategy in which all possible connections are soft-wired by genetics and then allowed to compete so that the "best" or most fit end up making the sole final connections. Polyinnervation at the end plate zone then allows motor neuron processes to interact in physical proximity with their direct competitors. But how is the competition decided? What defines the "best" connections?

A simple naive view of synapse formation and elimination proposes two hypotheses, one for each process. The first is that the muscle fibers and motor neurons to a developing muscle form two pools which are initially wired together at random. That is, each motor neuron would have the same probability as any other motor neuron of innervating any particular muscle fiber. In the rabbit soleus, the preparation used for much of this thesis, that probability in the neonate is approximately 4% given a fourfold polyinnervation by roughly 100 motor neurons on roughly 10,000 muscle fibers. If there were no preference for denervated muscle fibers in the formation of connections, then according to binomial statistics 98.2% of the fibers would be innervated. The few remaining uninnervated fibers could easily go undetected.

The second naive hypothesis then proposes that redundant synapses are lost at random and no further changes occur in the wiring once monoinnervation has been achieved. This hypothesis is not entirely naive because the last synapse on a muscle fiber is always retained. If synapses were lost strictly at random until 10,000 synapses remained in a muscle with 10,000 muscle fibers, then again according to binomial statistics fully 37% of the fibers would be denervated, when in fact denervated end plates are never seen.

One scenario befitting the naive hypotheses sees the initial polyinnervation as an accident of development. It is known that inactive muscle fibers at all stages, even in embryos, cause motor axons to sprout processes (Brown, Holland, & Hopkins, 1981; Ziskind-Conhaim & Bennett, 1982). Perhaps developing muscle fibers perfume their surroundings with some sort of factor (Brown, Holland, Hopkins, & Keynes, 1981) or appropriately label the basal lamina of their end plates with a non-diffusible factor as lures to bring in neuronal inputs. One might think that once a synapse had been obtained, the lure could be turned off as a guard against the potential complications of bigamy. There's no need to flirt purposely with multiple suitors if one is sufficient, but perhaps there is a time lag in turning the lure off or there is insufficient feedback from a single tentative embryonic synapse. By deliberately slowing down the feedback, the muscle fiber might be trying to draw a firm commitment out of one of its suitors. There need be no other reason for the polyinnervation than to guarantee a stable input to every muscle fiber. The price for that guarantee would then be a period of wholesale synapse elimination around the time of birth in order to reduce to one the number of inputs to a muscle fiber. Polyinnervation might be an unintended but unavoidable consequence of mechanisms designed to ensure reliable connections between motor neurons and muscle fibers.

Polyinnervation and synapse elimination in muscle may alternatively provide an opportunity for active competition between motor neurons for muscle fibers, but to date the strongest argument for that possibility lies in the analogy to development in visual cortex. There, a prenatal overproduction of overlapping inputs is sculpted by postnatal experience into a segregated pattern of ocular dominance columns (Hubel, Wiesel, & LeVay,

1977; Shatz & Stryker, 1978; LeVay, Stryker, & Shatz, 1978). Without visual experience, the columns do not develop nearly so clearly (Stryker, 1981; Swindale, 1981). Is there a similar reorganization coincident with synapse elimination in muscle? The analysis of motor unit properties in this thesis reveals several enticingly nonrandom aspects of neuromuscular development.

Summary of Thesis

The random innervation proposed by the first naive hypothesis would generate a Gaussian distribution of motor unit sizes in the embryo or neonate. For the rabbit soleus muscle studied herein the prediction for such a distribution would be an average motor unit size of 400 muscle fibers per motor neuron with a standard deviation of only 5%. In fact, peak motor unit twitch tensions (a measure of motor unit size) in the neonate showed considerable variability in size, corresponding to 50-1500 muscle fibers per motor neuron (Fig. 1 and Figs. 4 and 5 of Chapter 3). Thus, contrary to the naive hypothesis, neonatal polyinnervation, and perhaps the original innervation as well, is not random with respect to number of muscle fibers innervated by each motor neuron. Motor neurons may for example differ in their access to muscle fibers on the basis of their time of entry into the developing muscle mass. The first to arrive may find more synaptic sites available and thereby form larger motor units.

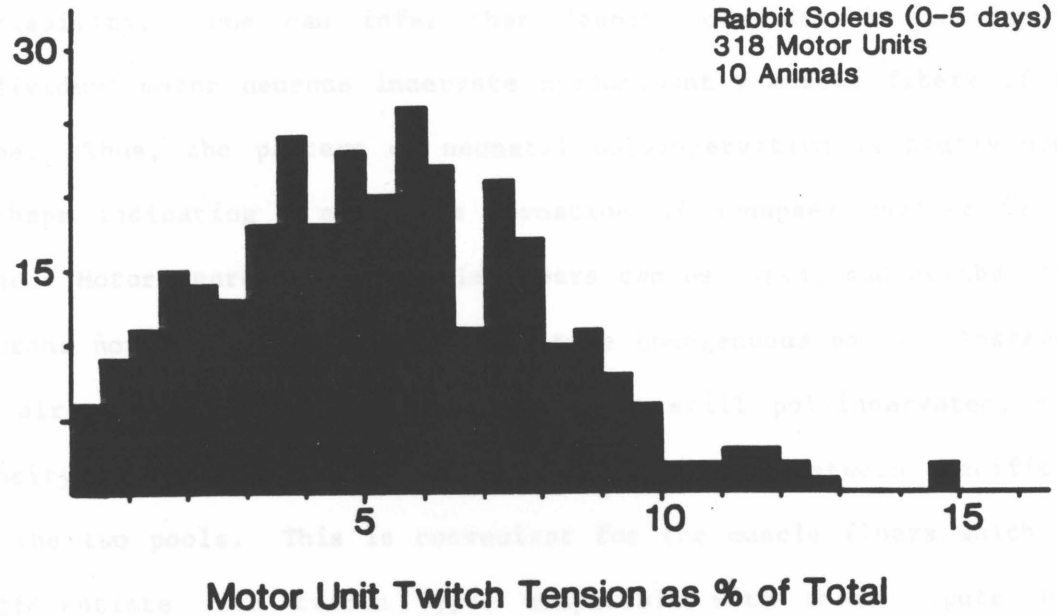
Neonatal rabbit soleus muscle fibers show different histochemical profiles for ATPase. If the original innervation were random with respect to muscle fiber type, then every motor neuron would innervate similar fractions of each fiber type. Every motor unit would thus show the same

Figure 1

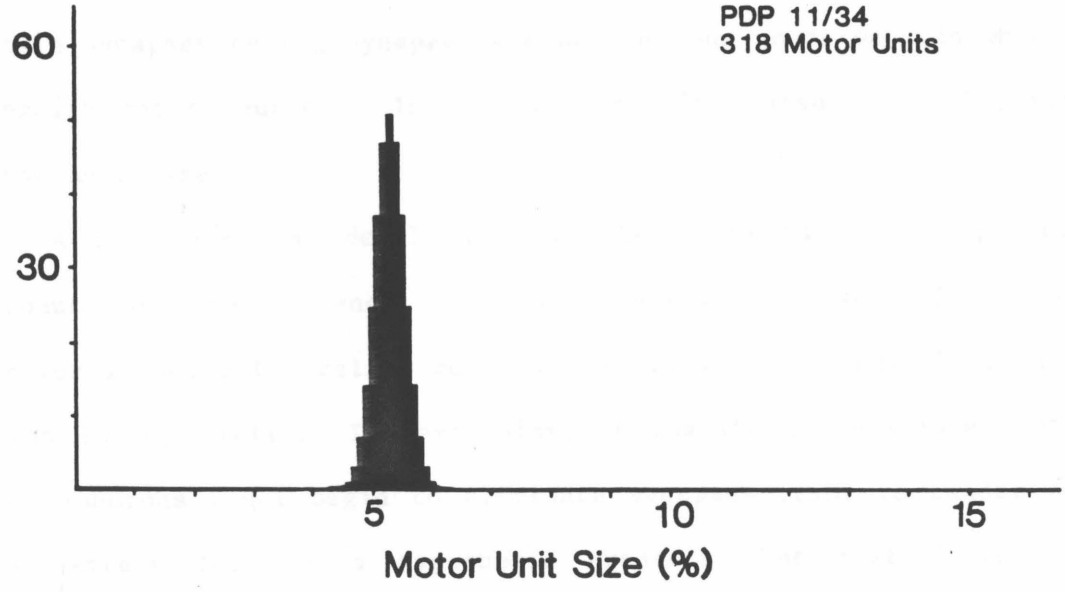
(a) Pooled distribution of 318 soleus motor unit peak twitch tensions from 10 rabbits aged 0 to 5 days old (from Fig. 1 of Chapter 3).

(b) Distribution of 318 motor unit sizes with the same average tension as the pool in (a) simulated by a random formation of synapses. Note that the simulated distribution is considerably narrower than the observed distribution in (a). As is shown in Figs. 5 and 6 of Chapter 3, the observed distribution is quite broad even after taking into account contributions to the diversity from differences in average motor unit twitch tensions between animals and from differences in average motor unit twitch tensions between fast and slow motor units.

NEONATAL DISTRIBUTION OF MOTOR UNIT TENSIONS



SIMULATED NEONATAL DISTRIBUTION



twitch time course, an admixture of fast and slow fiber responses. However, in the neonatal rabbit soleus muscle, I found very marked differences in twitch time courses which are not compatible with random variability. One can infer that despite considerable polyinnervation, individual motor neurons innervate predominantly muscle fibers of a single type. Thus, the pattern of neonatal polyinnervation is highly nonrandom, perhaps indicating a nonrandom formation of synapses earlier in development. Motor neurons like muscle fibers can be typed, and neither the motor neurons nor the muscle fibers constitute homogeneous pools. Instead, there is already at birth, while the muscle is still polyinnervated, a heterogeneity in both pools and a highly ordered wiring between specific subsets of the two pools. This is convenient for the muscle fibers which can then differentiate into twitch types compatible with their inputs despite a potentially confusing state of polyinnervation.

The naive hypotheses proposed a random elimination of redundant synapses, as well as a random formation. The alternative is that there is a nonrandom competition between motor neurons for synaptic sites within the muscle. To determine whether motor neurons differ in their ability to retain synapses during synapse elimination, one needs ways in which to distinguish motor neurons. In this thesis, I've pursued spinal position and motor unit sizes.

A rostral/caudal development of the spine has been hypothesized to account for the tendency of singly innervated muscle fibers to occur earlier in several forelimb muscles than in several hindlimb muscles (Bixby & Van Essen, 1979a). In particular, it was thought possible that rostral motor neurons might begin to eliminate synapses prior to caudal ones. In its extreme form, this hypothesis predicts that rostral motor neurons

within a single motor pool might begin eliminating synapses before caudal motor neurons within the same pool. The rostral motor neurons then would be at a competitive disadvantage and ultimately would lose more synapses than caudal motor neurons. In support of this proposal, Miyata and Yoshioka (1980a,b) claimed that synapse elimination in the rat soleus occurred exclusively among motor neurons of the more rostral of the two contributing spinal roots.

I have reexamined the possibility that elimination varies with spinal segment in both the rabbit and rat soleus by comparing average motor unit twitch tensions for the contributing spinal segments. In both species, synapse elimination proceeded to the same extent in all spinal segments. The discrepancy between my findings and those of the previous workers can be explained by technical problems with the earlier assay. Thus, synapse elimination can be considered as random with respect to spinal position, at least in the soleus muscle of these two species. Are there other ways, however, in which to distinguish the competitive abilities of motor neurons?

Motor neurons can be distinguished on the basis of the number of muscle fibers which they innervate. Brown et al. (1976) hypothesized that every motor neuron in the soleus motor pool had an equivalent potential to maintain synapses. In the competition for survival during synapse elimination, the terminals of small motor units were thus at a selective advantage relative to those of large motor units. On the basis of this hypothesis, the relative distribution of motor unit sizes should be narrower following the loss of polyinnervation than it was before. However, I have found that when one looks separately at populations of fast or slow motor units in the

rat soleus, there are small but consistent increases in the diversity of apparent motor unit sizes during the loss of polyinnervation.

Two dramatic observations indicate that a secondary reorganization of synapses occurs in the rabbit soleus muscle following two weeks of age, a time after which muscle fibers receive predominantly one input. First, ATPase histochemistry reveals that muscle fiber types continue to differentiate such that 2/3 of the fibers at two weeks of age are fast, but only 1/3 are fast by 5 to 6 weeks of age. During the same time period, the relative incidence of fast and slow motor units does not change markedly. Furthermore, the bulk of the transformation occurs on the ventral side of the muscle, yielding a marked spatial gradient in a matter of a few weeks. Second, over the same period of time, slow motor unit twitch tensions become larger relative to fast motor unit twitch tensions, yet the cross-sectional areas of slow fibers grow relatively less than do those of fast fibers. These two lines of evidence are consistent with the possibility that between 2 and 5 weeks of age, axons from slow motor neurons sprout to take synaptic sites away from fast motor neurons, especially in the ventral half of the muscle.

The role of activity in the elimination of neuromuscular synapses has been considered in several reports during the past decade. Blocking neuromuscular activity delays the elimination of synapses (Benoit & Changeux, 1978; Thompson, Kuffler, & Jansen, 1979) while increasing activity advances it (O'Brien, Ostberg, & Vrbova, 1978; Thompson, 1983a). Does activity, however, play a role in the competition between terminals at neuromuscular end plates? Are active synapses better able to retain synaptic sites than inactive ones during the loss of polyinnervation? It was possible to address this question by blocking activity for 4 or 5 days in a spinal root

that makes a minor contribution to the rabbit soleus at a time near the end of the period of polyinnervation. Preliminary results suggest that synapses inactivated for several days are at most at a small disadvantage relative to active synapses in competing for synaptic sites on active muscle fibers.

In summary, I report in this thesis that the innervation of the rabbit soleus muscle as seen at birth appears to be nonrandom with respect to the number and type of muscle fibers innervated by individual motor neurons. Some motor neurons preferentially innervate fast muscle fibers while others preferentially innervate slow muscle fibers. Even within each class, some motor neurons seem better able to make synapses than are others. The ensuing elimination of redundant synapses is random with respect to spinal position and to a large extent with respect to motor unit size as well. The level of neuronal activity *per se* probably plays no more than a minor role in the choice of which synapses are eliminated. Following the loss of redundant innervation, motor units reorganize such that muscle fibers originally innervated by fast motor neurons and appearing predominantly in the ventral region of the muscle lose their inputs and become preferentially innervated by slow motor neurons. Thus, I have found evidence for distinctly nonrandom patterns both in the neonatal polyinnervation and in a synaptic reorganization following the elimination of polyinnervation. However, I have found little evidence for a differential loss of synapses during the elimination of polyinnervation.

METHODS

Experiments were conducted on the soleus muscles of male and female New Zealand White rabbits (ABC Rabbitry, Pomona, CA) aged from fetal day 26 (4 to 6 days before birth) to postnatal day 43 and on Sprague Dawley rats (Simonsen Laboratories, Inc., Gilroy, CA) aged from birth to postnatal day 43. The soleus was chosen for a combination of practical and historical reasons. Structurally, it is a relatively simple muscle with muscle fibers running the length of the muscle in parallel and inserting into parallel tendons at both ends. In many species (e.g., rat, cat, and human), the adult soleus consists largely or exclusively of slow fibers which are grouped into motor units whose sizes are relatively less diverse than those of other muscles (McPhedran, Wuerker, & Henneman, 1965; Close, 1967; Bagust, 1972). The development of the soleus, furthermore, has been extensively studied in both rat and cat (Hammarburg & Kellerth, 1975a,b; Kugelberg, 1976; Bagust, Lewis, & Westerman, 1974).

The rabbit was originally of interest because of its larger size at birth (60 gm) than the rat (10 gm) and the availability of neonates. Experiments requiring invasive manipulation, such as those involving activity blocks as described in the Appendix, while impossible in the neonatal rat for reasons of scale and fragility, were approachable in the bunny. The rabbit soleus has proven an advantageous system in other ways as well. In particular, the number of muscle fibers and contributing motor neurons, in the small number of cases examined, seems to be constant after birth (Bixby & Van Essen, 1979a; Bixby, Maunsell, & Van Essen, 1980). In addition to the direct counts of muscle fiber number, I have found no evidence for forming or degenerating muscle fibers in cross sections of postnatal rabbit soleus muscle, at least after 5 days of age. Prior to this age, muscle fibers are so small that forming or degenerating profiles

could not always be distinguished. Westerman et al. (1973) have similarly found that muscle fiber and motor neuron numbers are constant after birth in cat soleus. The rat soleus, in contrast, seems to undergo a limited amount of myogenesis after birth (Chiakulas & Pauly, 1965; Riley, 1977; Thompson & Jansen, 1977), though the number of motor neurons appears to remain constant (Brown, Jansen, & Van Essen, 1976). Thus, in the rat soleus, there may be a period after birth in which synapse elimination occurs simultaneously with synapse formation, a potentially complicating factor. The rabbit soleus is of additional interest because the adult muscle contains many type II (fast twitch) as well as the predominant type I (slow twitch) muscle fibers (Guth & Samaha, 1969; Brooke & Kaiser, 1970). Issues of comparative development between distinguishable motor units can thus be and are herein addressed in this system.

The development of rabbit and rat soleus muscles was divided into three basic time periods for the purposes of this thesis (see Table): early (1-5 days of age; a.k.a. "neonatal"), corresponding to a period of extensive polyinnervation (with an average of three inputs per muscle fiber); intermediate (11-18 days of age), corresponding to a period just after wholesale loss of polyinnervation; and late (35-43 days of age), corresponding to a near adult state of affairs. Developmental ages were calculated relative to the time of birth, which occurred between 30 and 32 days of gestation. Animals thus aged varied considerably in weight (up to twofold at any age), and their muscles varied up to twofold in weight and absolute maximal tension. It may be that age from birth was at variance with developmental age by one or two days. Such variability in developmental age could contribute to differences in apparent degree of polyinnervation in the early group as reflected in differences in average

TABLE: DATA SUMMARY

polyinnervation ——————.....
single innervation .....——————
full complement of motor neurons ——————————————
full complement of muscle fibers .....——————————

	relevant chapters	ed. 26	pn. 1-5	6-10	11-18	denervated (13.5)	part. den. (11-16)	20-32	36-48	adult
Physiology of Motor Units (using bimorph & hand measurements from oscilloscope)	Intro, 2, Appen.		10 (318)		7 (236) 10 (220)*				6 (112)*	
Physiology of Motor Units (using AME DC force transducer and computer digitization)	1,2,3	1	9 (243)		8 (246)		8 (109)		4 (92)*	
Intracellular Physiology of end plate potentials	3				2			5		
ATPase Histochemistry	1,3	1	5	3	5	1	1		6	1
Silver Stain	3		3	2	14			1	7	

Each table entry gives the number of animals examined at a particular age with a particular technique. Numbers in parentheses for the physiology give the number of motor units recorded. *indicates venous perfusion/soleus nerve split paradigm (see text).

motor unit twitch tensions. Some variability in muscle fiber size and histochemical differentiation in age-matched muscles was also observed. Even within a single litter, significant variability was found in the various measure of developmental age, indicating that variability in the length of gestation was not the sole factor contributing to the differences. In any event, the division of the development into three wide and well separated time windows rendered such variability in age unimportant. Furthermore, physiological characteristics of the muscles were scaled to whole muscle properties so as to facilitate comparisons between differently sized muscles at a given age and between ages as well.

In Vitro Physiology

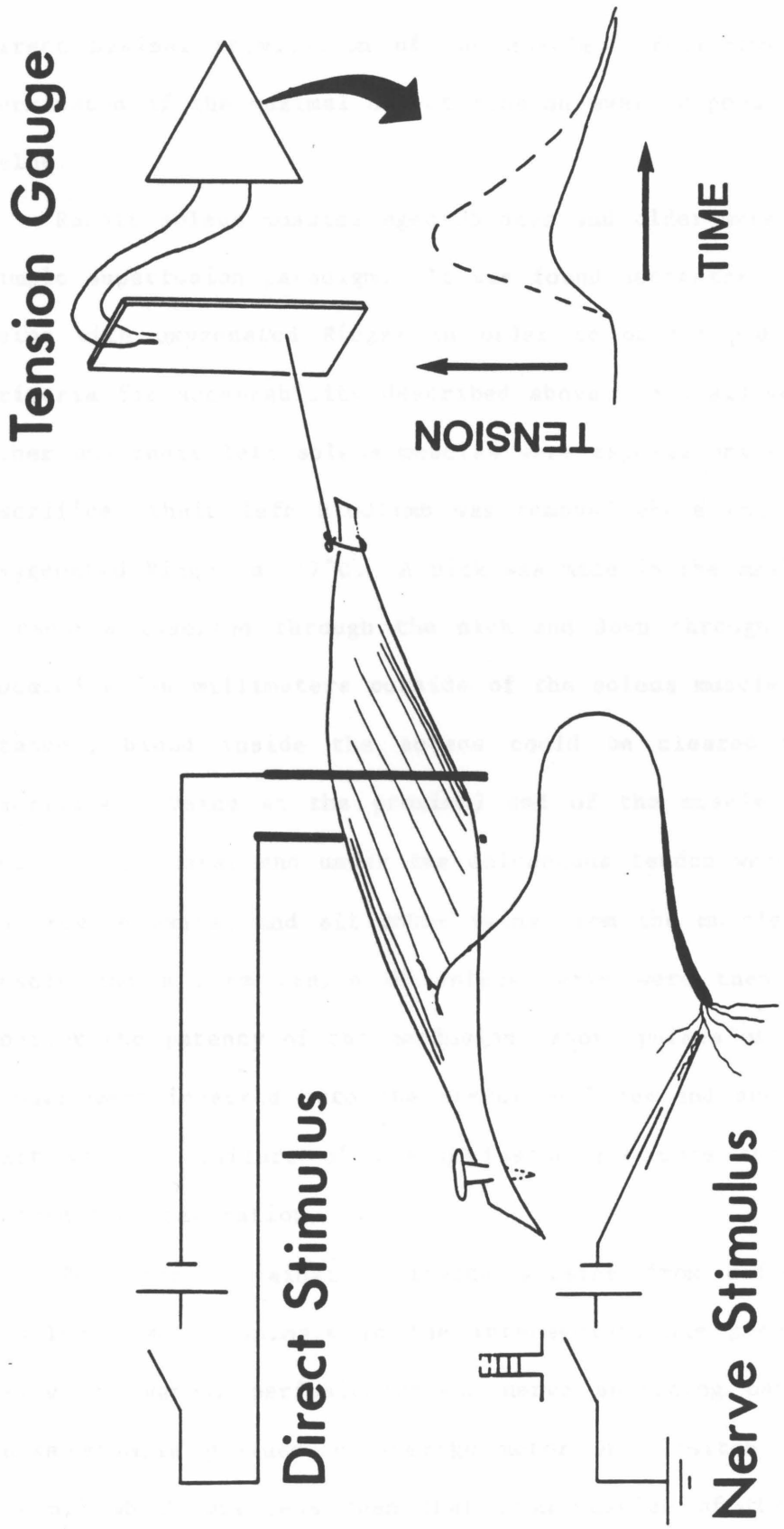
The physiology of all muscles was examined *in vitro* as pioneered for neonatal mammalian muscle by Liley (1956). While the muscles were thus deprived of natural hormones and their usual source of oxygen, evidence is presented below that the motor axons and muscle fibers were fully responsive. Furthermore, one must consider that muscles studied in the classical *in vivo* paradigm (Burke, 1981) are subject to the effects of anesthesia, both direct and indirect. Many of the *in vitro* physiology results for 5 to 6 week old rabbit soleus were in agreement with the earlier *in vivo* results for adult rabbit reported by Bagust (1972, 1979). In particular, the average motor unit twitch tension as a percentage of whole muscle twitch tension, the overall distribution of motor unit twitch tensions, and the motor unit twitch times-to-peak showed good correspondence between the two preparations. Motor units in the *in vitro*, 5 to 6 week old rabbit soleus preparation averaged 1.35% and 2.35% in twitch tension in two different

series of experiments. Those from the *in vivo* adult rabbit soleus averaged 1.0%. The twitch times-to-peak in both preparations varied considerably, being threefold in 5 to 6 week old bunnies, and fully sixfold in adults.

Following ether anesthesia and sacrifice, muscles were dissected free along with their contributing nerve. In neonatal and most intermediate aged preparations, dissections were continued through the spinal roots (usually S1 and S2, but sometimes also L7 and S3 in the rabbit and always L4 and L5 in the rat). They were then pinned via their fibular attachments to the bottom of a wax lined glass chamber, and their calcaneous tendons were tied with 6-0 surgical silk to a sensitive quasi-isometric force transducer (Fig. 1). Throughout, muscles were superfused with oxygenated Ringer solution containing 150 mM Na, 5 mM K, 1 mM Mg, 5 mM Ca, 167 mM Cl, 16 mM glucose, and 4 or 14 mM Hepes buffer. Bipolar silver electrodes placed on either side of the muscle were used to stimulate whole muscle contractions with pulses measured in the bath at 20-40 V amplitude and 1-2 msec duration. The muscle was stretched to the center of a range of lengths giving maximal whole muscle contractions and left at that length throughout an experiment. Every time the optimal length was reexamined later in an experiment, it was found not to have changed. Bagust (1972) found that for every one of the 31 motor units examined in the adult rabbit soleus, the bias length which produced a maximal twitch tension was within 2 mm of the optimal bias length for the whole muscle. For the examples presented, the worst error introduced by a mismatch between optimal whole muscle and motor unit twitch tensions would be only 15%. Thus, even units with optimal lengths slightly different than that of the whole muscle (Lewis, Luck, & Knott, 1972; Bagust, 1971) would not have their tensions seriously degraded. Preparations were considered viable only if whole

Figure 1

Schematic of physiology paradigm. Whole muscle and motor unit twitch tensions were measured *in vitro* using a force transducer whose output was recorded on an oscilloscope or by computer. Whole muscle tensions were elicited directly by stimulation with silver bipolar electrodes, and individual motor unit tensions were elicited by stimulation of motor axons drawn into a fine suction electrode.


Tension Gauge
TENSION
TIME
Direct Stimulus
Nerve Stimulus

nerve elicited tensions gave identical contractions to tensions obtained by direct maximal stimulation of the muscle. Furthermore, experiments were terminated if the maximal direct tension ever dropped 15% from its original value.

Rabbit soleus muscles aged 35 days and older were not viable under the simple superfusion paradigm. It was found necessary to reverse perfuse the veins with oxygenated Ringer in order to obtain preparations meeting the criteria for acceptability described above. Animals were anesthetized with ether and their left soleus muscles were exposed prior to sacrifice. After sacrifice, their left hindlimb was removed above the knee and immersed in oxygenated Ringer at 17°C. A nick was made in the small saphenous vein and a cannula inserted through the nick and down through a valve consistently located a few millimeters outside of the soleus muscle. In optimal circumstances, blood inside the soleus could be cleared within 90 seconds of sacrifice. Veins at the proximal end of the muscle near the nerve entry and at the distal end under the calcaneous tendon were cut halfway through to provide exits, and all other veins from the muscle were tied off. The muscle and a 2 cm length of soleus nerve were then dissected free. To monitor the patency of the perfusion, short pulses of 1% Chikagoblau dye in Ringer were injected into the perfusion lines and seen to pass through the exit veins. Failure of the perfusion for more than a minute typically ruined the preparation.

To control against artifacts arising from differences in paradigm, muscles from 10 animals in the intermediate age group were also examined using the venous perfusion/soleus nerve splitting paradigm. Muscles from these animals yielded an average motor unit twitch tension (1.3% ± 0.07% s.e.m.) which was less than that from muscles of the same age which were

simply superfused (1.93% ± 0.12% s.e.m. in an early series and 1.62% ± 0.08% s.e.m. in a later series) (see Table). There were noticeable differences in the diversity of motor unit twitch tensions as well. The distribution from perfused muscles was described by a quartile ratio, a measure of diversity explained in Chapter 3, of 2.9 while the earlier series using a bimorph force transducer gave a quartile ratio for a comparable age group of 4.9, and a later series using a piezoresistive transducer gave a quartile ratio of 3.5. Fortunately, the differences between these three series of data were not important for the interpretations presented in this thesis. It may be that the differences reflected individual differences between the animals used in the three series in terms of number of motor units per soleus muscle and relative numbers of fast and slow twitch muscle fibers (Komi et al., 1977).

The temperature was maintained in the range 18 to 20°C and was held constant in individual experiments to within 0.4 C°. Temperatures much above or below this range caused premature death of the preparation or conduction block respectively. In some unacceptable experiments, temperature changes of 2 C° affected the magnitude and time-to-peak of whole muscle twitch tensions by as much as 10%. Because of the tight control on the temperature of the perfusion bath, variability due to temperature changes within an experiment was not present in the data reported here. Variability in physiological measurements between experiments due to differences in temperature of up to 2 C° were avoided in analysis by scaling on an experiment basis before pooling data. The scaling techniques are discussed in the appropriate results sections.

The force transducer in early experiments was a piezoelectric bimorph. Most of the experiments of this thesis, and all of those

involving temporal measurements, however, were conducted with either of two piezoresistive units for force ranges of 0-6 gm and 0-100 gm (Aksjeselskapet Mikro-Elektronikk, Horten, Norway, model AE 875). A regulated battery/op amp driver of in-house design in conjunction with these units gave linear responses down to the lowest calibration of 25 mg. Residual noise in the driving circuitry and Grass P16 DC recording amplifier limited the resolution of the 100 gm gauge to 3 mg.

All force transducers used were nominally isometric. However, they all function by converting displacement of a stiff member into a measure of force. For very small muscles, the displacement of the transducer could amount to 5% of the length of the muscle and may thus have somewhat degraded the twitch tensions from young muscles.

Twitch tensions of individual motor units were determined by splitting the ventral roots from spinal segments L7 through S3 as appropriate in the rabbit and segments L4 and L5 in the rat. In muscles sustained on reverse venous perfusion, only the soleus nerve stump was available, so it was painstakingly desheathed and split instead. Filaments were drawn into a glass suction electrode (200 μm tip opening) and stimulated with pulses of 1-9 V amplitude and 0.10-0.25 msec duration at intervals of 4 sec or greater. A minimum number of repetitions of motor unit stimulation were made at low frequency so as to avoid as much as possible fatigue of motor unit properties. When more than one soleus motor axon were present in filaments too small to split, stimuli were delivered at recruitment thresholds, and the differences in peak twitch tensions were taken as individual motor unit tensions. In computer monitored experiments, twitch traces from successively recruited units were subtracted by the computer. Separate experiments demonstrated the legitimacy of such subtractions. On

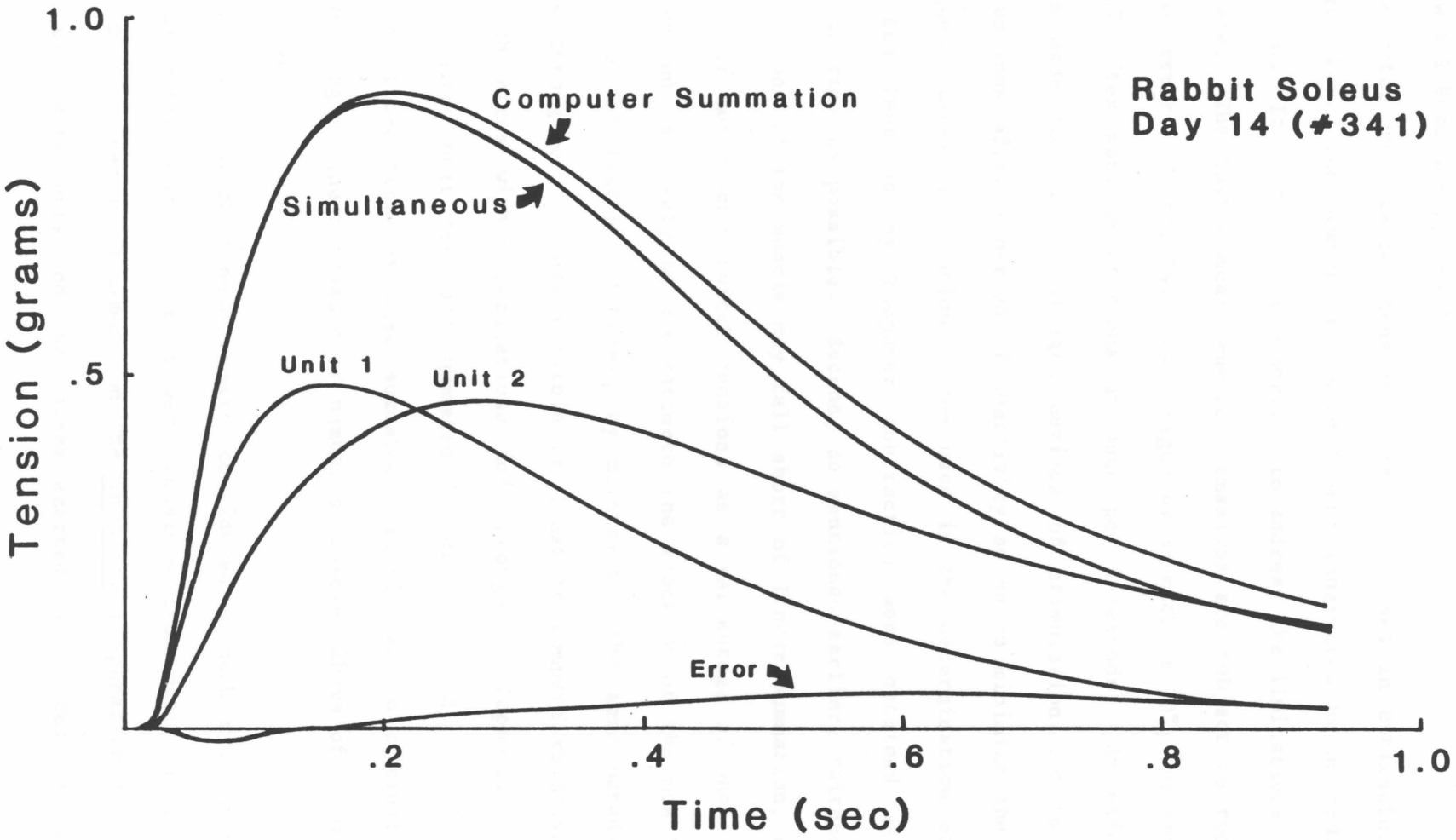
all 22 occasions where it was examined, twitch tensions of individual motor units were found to sum linearly in our preparations to within 15% (Fig. 2). Because of the potential complication of nonlinear summation of large twitches (Brown & Matthews, 1960), when more than four soleus motor axons were present in a single filament, the filament was either split further or discarded. Most of the data were obtained from filaments containing only one or two soleus motor axons.

Motor unit twitch traces were processed by computer and reduced to three descriptive parameters for further analysis: maximum tension, time to maximum tension after axonal stimulation, and time to half fall from maximal tension. Temporal measurements were scored relative to time of stimulation rather than relative to initial response because of difficulties in accurately resolving the latter. Delays between axonal stimulation and twitch response were 10 msec or less while times-to-peak twitch tension were greater than 100 msec. Motor unit twitch tensions were expressed as a percentage of the most recent maximal direct twitch tension. Such a normalization facilitated comparison of motor unit tensions between animals of the same age but different size and compensated for the changes of up to 15% in maximal direct tension tolerated during a physiology experiment. Of the two temporal parameters, twitch time-to-peak was preferable to time-to-half-fall as a measure of the motor unit type. Time-to-half-fall suffered from large variations with temperature and motor unit fatigue. Furthermore, times-to-peak split into a bimodal distribution reflecting fast and slow muscle fiber populations while times-to-half-fall gave a much less clean separation (also seen <u>in vivo</u> by Bagust, 1972). Times-to-peak for individual motor unit twitches were typically shorter

Figure 2

Summation properties of motor unit twitches in a 14 day old rabbit soleus (#341). Two motor units were stimulated individually and then simultaneously. The individual twitch traces were then summed by computer and the result compared with the simultaneous twitch. An error trace showing the difference between simultaneous and computer summated twitches amounts to 2% at the time-to-peak of the simultaneous twitch.

Summation Properties of Motor Unit Twitches



than for whole muscle twitches, a characteristic of *in vivo* preparations as well (Lewis & Rosendorff, 1965).

Peak motor unit twitch tensions were to be used in estimating motor unit size, i.e., the number of muscle fibers innervated by an individual motor neuron. It is therefore important to address the limitations in such an estimate. The twitch measurements themselves are subject to two basic sources of error. First, twitches fatigue by as much as 30%, but typically about 15% after many repetitions at once per 4 seconds. An effort was therefore made to avoid prolonged periods of stimulation and to record twitch tensions after a period of inactivity so as to minimize the amount of fatigue. Likewise, tension traces used in the determination of motor unit twitch tensions by computer subtraction were obtained as close together in time as possible. Second, as mentioned earlier, twitches from large fractions of the muscle may fall short of linear summation, so that expression of motor unit twitch tensions as a percentage of whole muscle twitch tension is likely to overestimate the fraction of the muscle contributing to the twitch. However, by maintaining the same paradigm and standards throughout, it was possible at least to compare relative motor unit twitch sizes within populations and through development. Because individual motor unit tensions summated linearly (Fig. 2), it is likely that muscle fiber tensions also summated linearly within a single motor unit, providing a linear measure of number of muscle fibers of a particular type innervated.

Motor unit twitch tensions were considered a much more convenient measure of motor unit size than were tensions from motor unit tetani. Twitches placed less of a demand on the *in vitro* preparation in terms of both energy requirements and the forces exerted on the delicate neonatal

fibular tendon. Twitch tensions were also more easily subtracted when motor units were subjected to the threshold stimulation paradigm. The efficiency of the twitch tension approach made it possible to routinely examine 25 and sometimes upwards of 50 motor units in the same preparation. In turn, the large number of motor units from individual preparations made possible cleaner comparisons between motor units and pooling of motor units between animals of the same age. Tetanus tensions in adult rabbit soleus, furthermore, were found by Bagust (1972) in an in vivo preparation to give a very similar distribution and average value as the twitch tensions of the same motor units. Individual twitch and tetanus values, however, did not necessarily correspond as twitch/tetanus ratios varied over a twofold range.

The remaining consideration in the assessment of motor unit size is the conversion from twitch tension to number of muscle fibers contributing to the twitch. The force elicited by a motor unit is given by the following equation (Burke, 1981):

Force = Innervation ratio x avg. area x specific tension

One can thus calculate the number of muscle fibers within a motor unit from the force elicited, the average cross-sectional area of the muscle fibers with a histochemical type corresponding to the motor unit's physiological type, and the specific tension or tension per unit area for the type of muscle fibers innervated. Unfortunately, the specific tension is a poorly understood factor and is virtually unstudied in developing muscle (Burke, 1981). Estimates of the ratio of specific tensions between fast motor units of medial gastrocnemius or flexor digitorum longus (FDL) and slow

soleus motor units in adult cat muscle, can vary from 1.4:1 to 2.2:1 (Burke, 1981). Within the FDL, the ratio between specific tensions for fast and slow motor units may be as high as 5.8:1, and it is not clear which is the better comparison. Furthermore, Close and Hoh (1968) found that decreasing the temperature from 37°C to 20°C increased the whole muscle twitch tension by 70% for adult rat extensor digitorum longus, a fast muscle, but not for soleus, a slow muscle. This raises the possibility that the ratio of specific tensions between fast and slow motor units, at the depressed temperatures used herein for *in vitro* physiology, may be even larger by 70%, i.e., up to 3.7:1 or even 8.3:1.

Anatomy

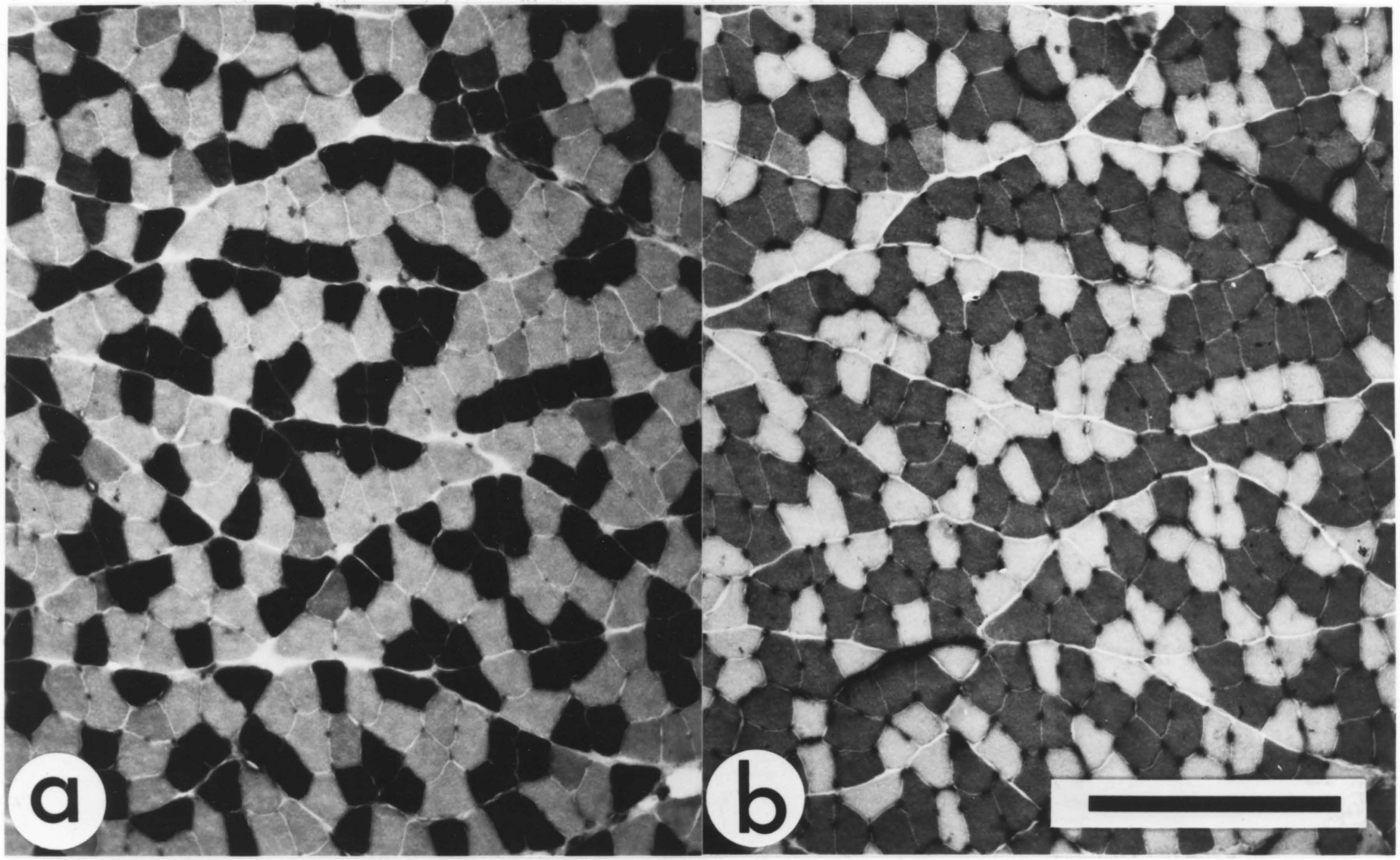
ATPase Histochemistry

Rabbit and rat soleus muscles in the same age groups used for motor unit physiology were examined for ATPase histochemistry according to the method of Guth and Samaha (1970). The basic chemistry of the reaction is that ATPase within muscle fibers breaks down the ATP in a reaction mixture into phosphate which at alkaline pH forms an *in situ* precipitate with calcium also present in the reaction mixture. The calcium in the precipitate is then replaced with cobalt, and in turn, the phosphate is replaced with sulfur to yield a black CoS reaction product.

The most reliable ATPase histochemistries were achieved with 10 μm cryostat-cut cross sections preincubated at pH 10.4 for 5 minutes followed by incubation at pH 9.4 with ATP and calcium for 30 minutes at 37°C (Fig. 3a). Dark profiles represent muscle fibers containing alkali stable ATPase and are interpreted as type II fibers corresponding to "fast twitch"

Figure 3

Adjacent cross sections through the soleus muscle of a 36 day old rabbit (#386) stained for ATPase. (a) Preincubated at pH 10.4 so that dark fibers correspond to type II, "fast twitch." (b) Preincubated at pH 4.10 so that dark fibers correspond to type I, "slow twitch." (Scale bar = 200 μm)


a
b

physiological properties. Similarly, light profiles represent muscle fibers containing alkaline labile (acid stable) ATPase and are interpreted as type I fibers corresponding to "slow twitch" physiological properties (Guth & Samaha, 1970; Burke, 1981). A minor fiber type stains with intermediate intensity and is interpreted as a subclass of type II.

Preincubation in acid buffer proved fickle with best results obtained for rabbit soleus on recently frozen, older muscles preincubated for 5 minutes at pH 4.10. When it worked, acid preincubation provided a complementary staining pattern to that seen for alkaline preincubation of adjacent sections (Fig. 3b). Only two fiber types are distinguished after acid preincubation. Those fibers which stain dark after pH 4.1 preincubation and with intermediate intensity after pH 10.4 preincubation are probably type IIC (Brooke & Kaiser, 1970; Kugelberg, 1976). The interpretation of ATPase fiber staining in young muscles is complicated (Guth & Samaha, 1972) and will be discussed in the next chapter.

Muscle fiber cross-sectional profiles stained for ATPase were photographed using a Zeiss Universal microscope on 4 x 5 Polaroid Type 55 Positive/Negative film. From the negatives, high resolution 8 x 10 prints were made which were then used for analysis of frequency and size of dark and light staining profiles in ventral, interior, and dorsal regions of muscles. Muscle fiber cross-sectional areas were determined by hand tracing of the photographed fiber profiles on a Wang model 662 digitizing tablet which fed into a computer algorithm.

Silver/Cholinesterase Stain

Terminal arborizations and end plates were visualized using the method of Namba, Nakamura, and Grob (1967) with modifications by W. G. Hopkins (1980 and personal communication). Freshly excised muscles were fixed and stored in buffered, osmotically balanced solutions containing dimethylsulfoxide (DMSO). Prior to staining, muscles were equilibrated in 30% sucrose, frozen, and sectioned longitudinally on a freezing microtome to thicknesses of 20-75 μm. Sections were stained either loose in vials or affixed to subbed slides. First, end plates were stained for cholinesterase following the method of Karnovsky and Roots (1964), and then the sections were dehydrated and rehydrated through stepped concentrations of ethanol. Neuronal processes were reacted for 30 minutes with borax buffered $AgNO_3$ and developed in a reducing agent containing 1% hydroquinone and 5% Na_2SO_3 (anhydrous).

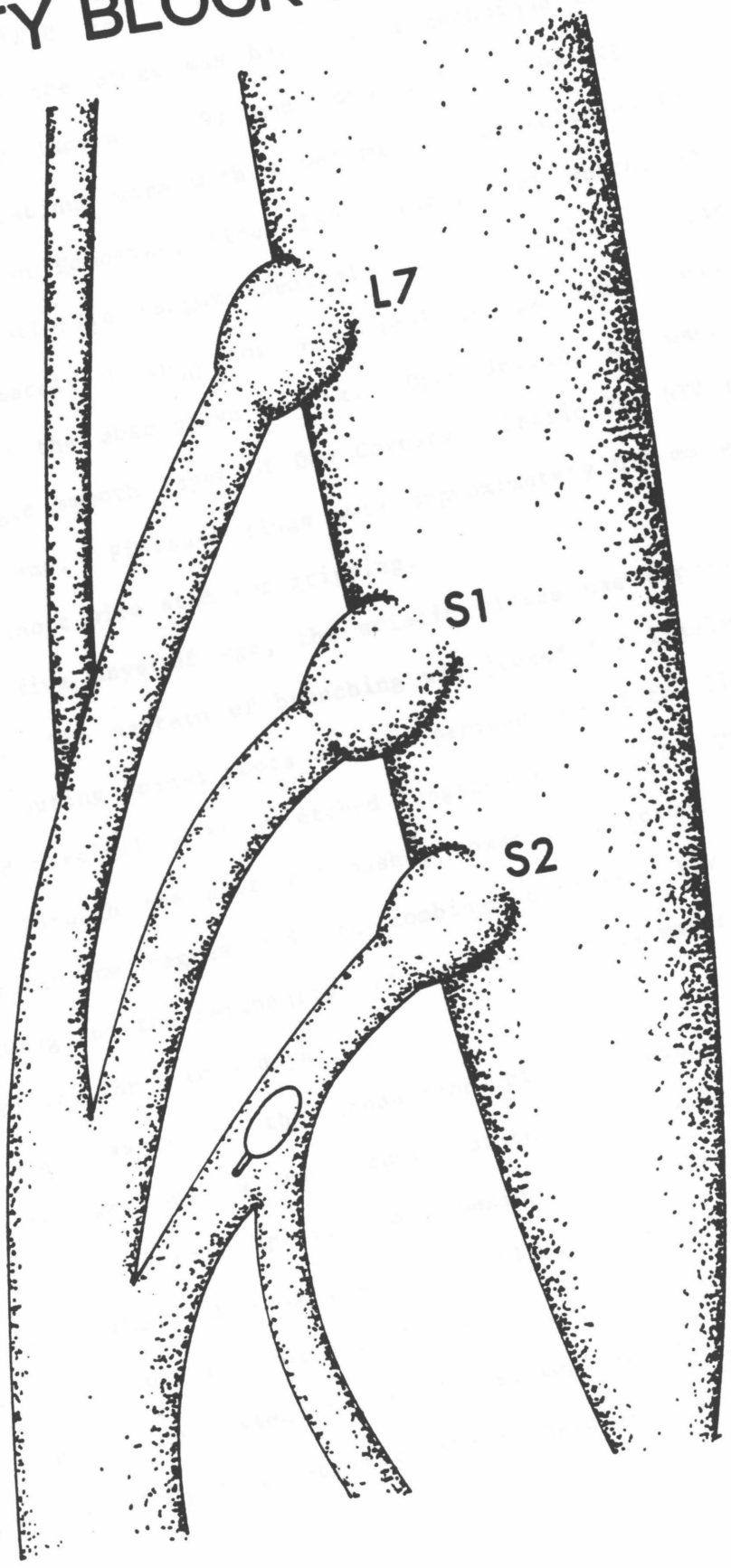
Nerve Block Paradigm

A method was devised to study the competitive success of active vs. inactive nerve terminals on active muscle fibers during the loss of polyinnervation in the rabbit soleus muscle. Two or occasionally three spinal roots (typically S1 and S2 but sometimes L7 or S3) contributed to the innervation of this muscle. From 4 or 5 to 8 to 10 days of age, a time when loss of polyinnervation is near its maximal rate in the rabbit soleus muscle (Bixby & Van Essen, 1979a), the activity in a spinal root making a minor contribution to the muscle could be blocked by surgical insertion of a small slowly-releasing plug containing tetrodotoxin (TTX, Sigma Chemical

Figure 4

Schematic diagram roughly to scale of a TTX plug implanted under the perineurium of spinal root S2 prior to its merger into the sciatic plexus. (Drawing by C. Talland.)

ACTIVITY BLOCK PARADIGM



Co., St. Louis, MO) beneath the perineurium of that root prior to its entry into the sciatic plexus (Fig. 4).

The manufacture of the plugs was based on a technique developed by Thompson, Kuffler, and Jansen (1979) and consisted of coating 40 gauge platinum/iridium or platinum wire with a wet mixture of collagen (Avitene Microfibrillar Collagen Hemostat, Alcon Labs, Inc., Fort Worth, TX) and alpha-cellulose microfibrils (Sigma Chemical Co.). A 0.2 µl droplet of 10 mM TTX (approximately 1 LD_{50} for a typical 100 gm bunny) was then allowed to dry into the absorptive matrix. Upon drying, the matrix was coated with a single smooth layer of Dow Corning Silastic 738 RTV thinned with anhydrous xylene. Finished plugs were approximately 0.5 mm wide and 3 mm long with a short wire stem for gripping.

At four or five days of age, the sciatic plexus was exposed from a dorsal approach. The pattern of branching was judged for likely dominant and minor contributing spinal roots and the perineurium of the likely minor root was opened carefully with an etched tungsten needle. The TTX plug was then inserted through the slit and pushed toward the spine. Within one day, the perineurium regrew and the combined diffusion barriers of the silastic coating and the perineurium served to routinely maintain blocks of neuronal activity for 4 to 5 days.

Behavioral assays of the conduction blocks proved futile as the blocked roots made only a small contribution to the musculature of the hindlimb. On occasion, spinal roots making large contributions to the innervation of the limb (e.g., L7) were blocked, resulting in behavioral deficits which could be monitored for at least 3 to 4 days. Furthermore, at the time of the dissection, the physiological efficacy of the blocks could be assayed by stimulation of the plugged root at a position between

the spine and the plug. Only those preparations in which a conduction block was ascertained at the time of dissection were used for further analysis. At this time only the plugged root showed a conduction block. Although it could not be checked in one and the same animal, it was felt that conduction blocks never spread to axons from other roots. First, contractions could frequently be elicited distal to the plug yet proximal to the junction with the sciatic plexus. Second, in a single control animal examined one day following the surgical implant, only the plugged root showed a conduction block. Upon verification of the activity block, the plug was removed and the preparation washed in Ringer's solution for 30 minutes prior to measurement of motor unit twitch tensions.

Chapter 1

SELECTIVE INNERVATION OF MUSCLE FIBER TYPES IN A DEVELOPMENTALLY POLYINNERVATED MUSCLE

Adult mammalian muscles consist of at least two types of muscle fibers, each innervated by a corresponding type of motor neuron (Burke, 1981). The muscle fiber types are distinguished by various histochemical reactions (Brooke & Kaiser, 1970), most notably actomyosin ATPase (Guth & Samaha, 1969), and by their physiological properties, particularly speed of contraction (Edstrom & Kugelberg, 1968). Motor neurons, in turn, can be distinguished by the physiological properties of the single type of muscle fibers which they innervate (Edstrom & Kugelberg, 1968) and by various correlated properties both physiological and anatomical (Burke, 1981).

All of the muscle fibers within an adult motor unit express the same histochemical and physiological properties (Edstrom & Kugelberg, 1968). These properties are subject to neural regulation, either by the pattern of activity (Salmons & Vrbova, 1969; Brown, 1973; Jolesz & Sreter, 1981) or by neural trophic factors (Karpati & Engel, 1967; Gutmann, 1976). During normal development, however, the neural control of muscle fiber differentiation is subject to a complication. Soon after formation, each myotube is transiently polyinnervated by several different motor neurons (Dennis, Ziskind-Conhaim, & Harris, 1981). Because of the potentially conflicting inductive influences from multiple inputs, it has been suggested that the differentiation into adult twitch types of muscle fibers or at least of various myosin components is postponed until single innervation has been achieved (Gauthier, Lowey, & Hobbs, 1978; Whalen et al., 1981).

Nonetheless, during polyinnervation, muscle fibers do differentiate into histochemically distinguishable types (Engel & Karpati, 1968; Riley, 1977; Kelly & Rubinstein, 1980; Sartore, Gorza, & Schiaffino, 1982). Three hypotheses could account for the early histochemical differentiation of muscle fibers. First, a muscle fiber might be genetically preprogrammed

toward a particular twitch type during development and subject to neural influences only later in life. Second, fiber type might be induced by a single dominant neural input, perhaps the first motor neuron to innervate (Rubinstein & Kelly, 1981). Third, individual muscle fibers might be polyinnervated predominantly by one or another type of motor neuron and thereby receive nonconflicting inductive influences.

The possible bases for the determination of muscle fiber type were addressed in this study with a physiological assay. Twitches were elicited by stimulation of single motor axons contributing to a developmentally polyinnervated muscle, the neonatal rabbit soleus. Twitches with distinctly different time courses were found, indicating a preferential innervation of particular muscle fiber types by particular motor neurons. Thus, in this muscle, at least some fibers show a physiological differentiation in accord with the observed histochemical differentiation. Furthermore, at least some motor neurons are differentiated into types as reflected in their preferential polyinnervation of particular muscle fiber types. That motor neurons of a particular type preferentially share end plates with other motor neurons of the same type allows for a neural induction of muscle fiber differentiation during early development. It also places constraints on the nature of the development of neuromuscular innervation.

Methods

The physiology of soleus motor units from New Zealand White rabbits was examined *in vitro* at 18-20°C. Twitch tensions in response to stimulation of individual motor axons were recorded by computer and subsequently

analyzed for peak tension as a percent of whole muscle peak tension and for time to peak. 243 motor unit twitch tensions were recorded from nine muscles of animals aged 1.5 to 4 days old; 246 motor unit twitch tensions were recorded from eight muscles of animals aged 11 to 16 days of age. Muscles were stained for ATPase histochemistry according to the method of Guth and Samaha (1970). (See Methods chapter for additional details.)

Results

Histochemistry

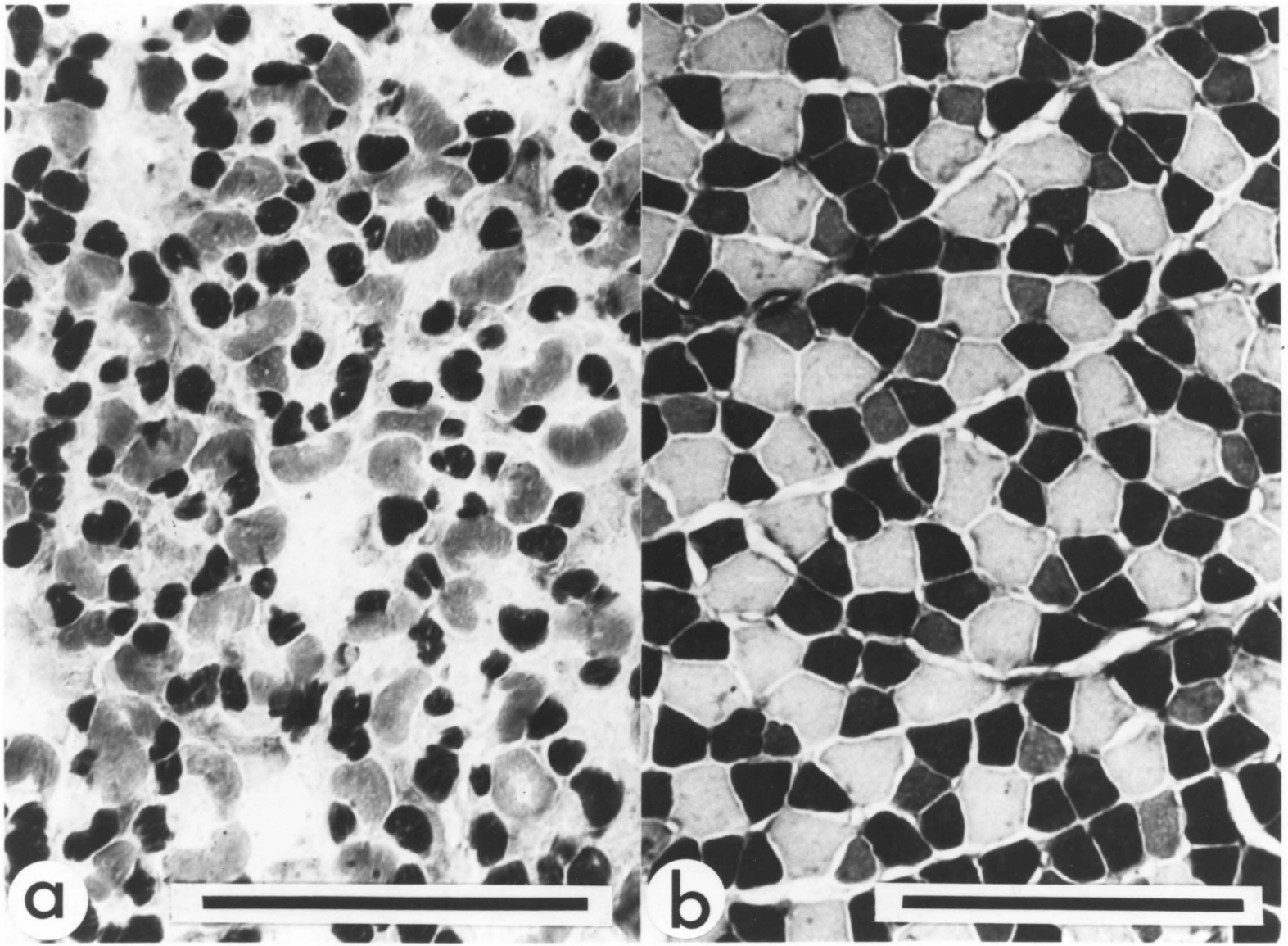
The soleus muscle of rabbits aged from birth to 5 days old is three- to fourfold polyinnervated with each motor neuron innervating about 400 muscle fibers on average (Bixby & Van Essen, 1979a, and Chapters 2 & 3). At the same stage in development, ATPase histochemistry revealed two anatomical classes of muscle fibers (Fig. 1a; Chapter 3). At about 14 or 15 days of age, following the loss of polyinnervation (Bixby & Van Essen, 1979a), three fiber types were distinguished by ATPase histochemistry (Fig. 1b; Chapter 3).

Motor Unit Twitch Time-to-Peak

If the projections of motor neurons were nonspecific with respect to the types of muscle fiber innervated, then, because of the large motor unit sizes, each motor axon would innervate very similar fractions of each muscle fiber type. Every motor unit would then show essentially the same twitch time course resulting from an admixture of fast and slow fiber responses. In contrast, the actual motor unit twitches from neonatal rabbit soleus muscles showed time courses nearly as disparate as those seen

Figure 1

ATPase histochemistry showing mixtures of fiber types in postnatal rabbit soleus muscle. Incubation at pH 9.4 following preincubation at pH 10.4 according to the method of Guth & Samaha (1970). (a) At 3 days of age, many of the muscle fibers are just exiting the myotube stage as judged by their centrally located nuclei (Guth & Samaha, 1972). (Scale bar = 100 μm.) Two fiber types are seen at this time. Type I fibers are large and lightly-staining (average cross-sectional area across muscle = 160 μm^2) while the type II fibers are smaller and darkly-staining (70 μm^2; Burke, 1981). It is likely that the former type represents primary slow-twitch fibers while the latter type represents secondary fast-twitch fibers (Burke, 1981; but see Guth & Samaha, 1972). (b) Three fiber types are seen at 15 days of age. (Scale bar = 100 μm.) The relative cross-sectional areas of the lightest (type I; 480 μm^2) and darkest (type II; 230 μm^2) staining fibers have changed little, but a third intermediate-staining fiber type is also seen (possible type IIC; 190 μm^2).


a
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in adult animals (Fig. 2). This suggests that in a polyinnervated muscle at least some motor neurons preferentially innervated predominantly either fast twitch or slow twitch muscle fibers. A plot of the twitch properties of 31 motor units recorded from a representative 4-day-old muscle revealed a clear disparity in motor unit physiologies (Fig. 3a). Some motor units produced typically small, slow twitches while others produced relatively large, fast twitches. Lying in a spectrum in-between were motor units with typically smaller twitches and fast to intermediate time courses. The diversity in times to peak of a twitch was as marked in the polyinnervated neonatal muscle as it was ten days later once elimination of the redundant synapses had occurred (Fig. 3b). In fact, all 17 muscles, both polyinnervated and singly innervated, showed a two- to threefold range of motor unit twitch times-to-peak (Fig. 4).

Embryonic Rabbit Soleus

A younger muscle with uniformly staining fiber profiles was examined to test whether histochemical differentiation is a necessary correlate of variability in motor unit twitch times-to-peak. In a single soleus muscle from an embryonic rabbit at 26 days of gestation (4 to 6 days prior to birth), muscle fibers were found to be histochemically undifferentiated. At the same time, motor unit twitch times-to-peak fell within only a 1.4-fold range compared to a 2.4-fold range on average in the nine neonatal muscles. Thus, there seems to be neither histochemical differentiation of muscle fibers nor physiological differentiation of motor units in the rabbit soleus muscle at 26 days of gestation. Between 26 days of gestation and birth it may still be possible to find physiological differentiation

Figure 2

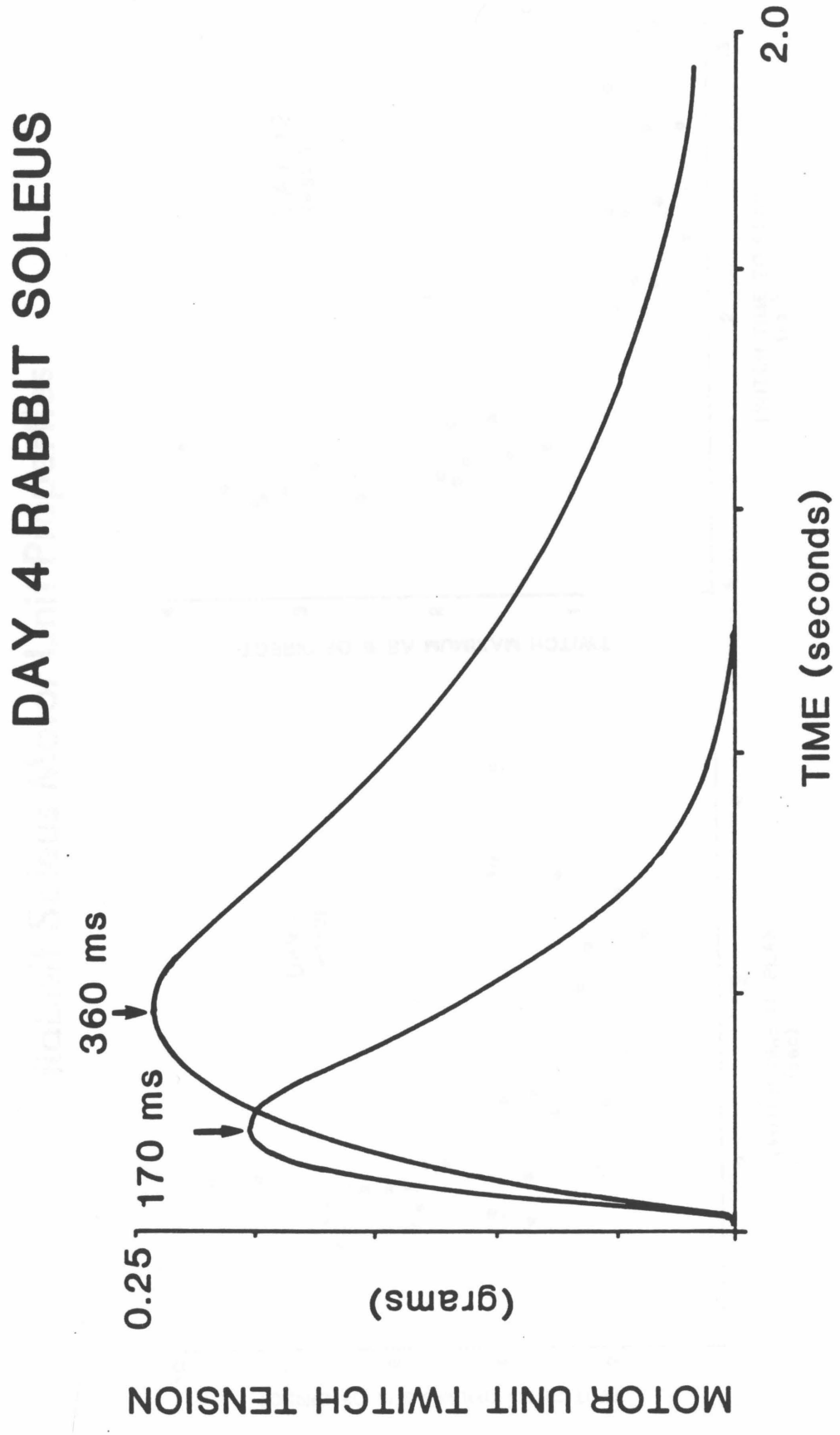
Fast and slow motor unit twitches from a 4-day-old rabbit recorded within 5 minutes of each other (animal #362, units 27 and 28). Note that the prolonged time scale is a result of the low temperatures (18-20°C) used for *in vitro* physiology.

Figure 3

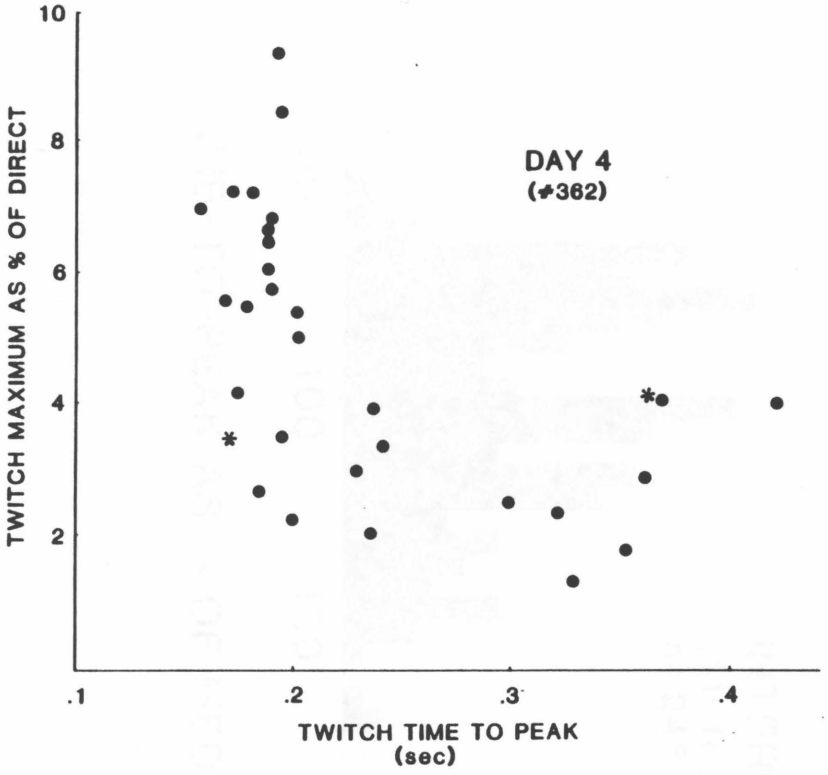
Motor unit twitch peak tensions vs. times-to-peak from representative animals. (a) Example of a 4-day-old animal (#362) with about two- to threefold polyinnervation. Asterisks mark the two twitches illustrated in Fig. 2. (b) Example of a 12-day-old animal (#319) with predominant single innervation.

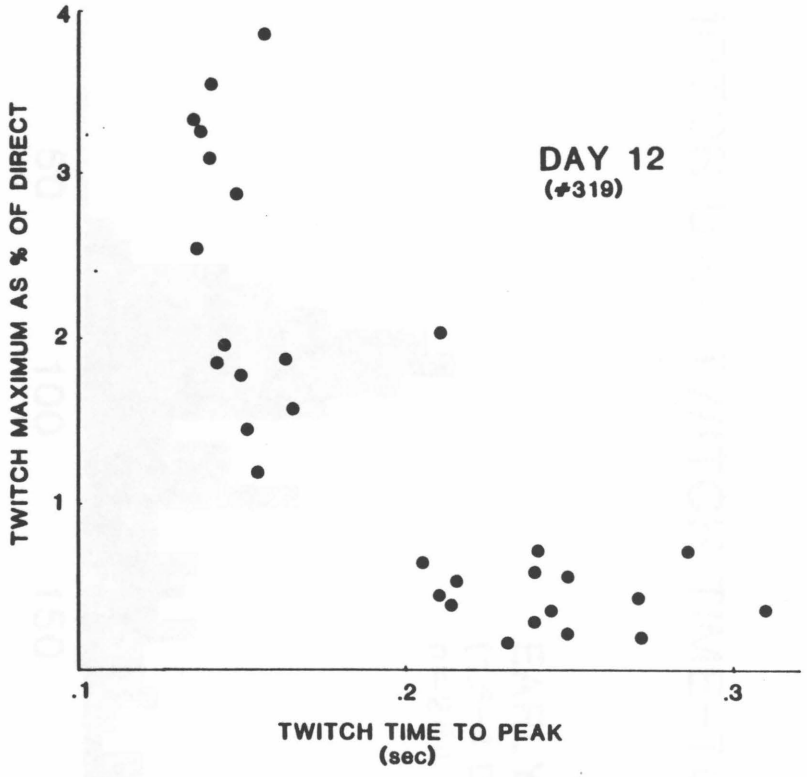
Figure 4

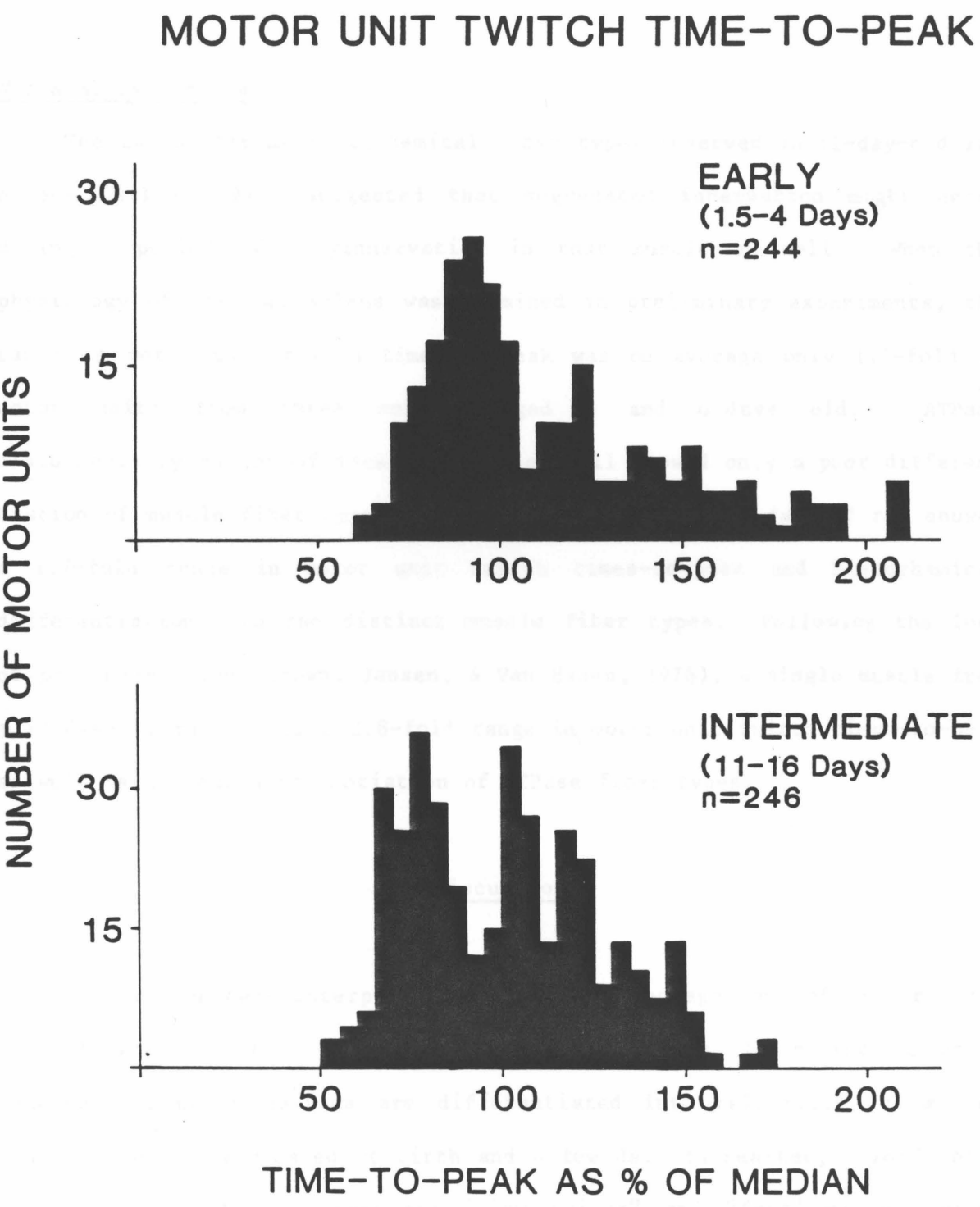
Motor unit twitch times-to-peak scaled to the median for each animal and pooled for two developmental age groups. (See also Fig. 2 of Chapter 3.)


DAY 4 RABBIT SOLEUS
MOTOR UNIT TWITCH TENSION
(grams)
0.25
170 ms
360 ms
TIME (seconds)
2.0

Rabbit Soleus Motor Unit Properties






MOTOR UNIT TWITCH TIME-TO-PEAK
EARLY
(1.5-4 Days)
n=244
INTERMEDIATE
(11-16 Days)
n=246
NUMBER OF MOTOR UNITS
30
15
50
100
150
200
TIME-TO-PEAK AS % OF MEDIAN

prior to histochemical differentiation. The following preliminary results from the developing rat soleus bear on this issue.

Neonatal Rat Soleus

The two different histochemical fiber types observed in 11-day-old rat soleus (Riley, 1977) suggested that segregated innervation might occur during a period of polyinnervation in that muscle as well. When the physiology of the rat soleus was examined in preliminary experiments, the range in motor unit twitch times-to-peak was on average only 1.2-fold in motor units from three animals aged 3 and 4 days old. ATPase histochemistry on one of these muscles as well showed only a poor differentiation of muscle fiber types. A single muscle from a 5-day-old rat showed a 1.7-fold range in motor unit twitch times-to-peak and histochemical differentiation into two distinct muscle fiber types. Following the loss of polyinnervation (Brown, Jansen, & Van Essen, 1976), a single muscle from a 17-day-old rat showed a 2.8-fold range in motor unit twitch times-to-peak as well as a clear differentiation of ATPase fiber types.

Discussion

A parsimonious interpretation of the segregation of motor unit physiologies in rabbit soleus is that early during the period of polyinnervation, motor neurons are differentiated into relatively "slow" and "fast" types. As assayed at birth and a few days thereafter, "slow" motor neurons preferentially innervate "slow twitch" and "fast" motor neurons preferentially innervate "fast twitch" muscle fibers. At a specific time

in development, then, axon terminals from motor neurons of the same type are found preferentially together on the same end plates.

Motor Unit Differentiation

The marked differences between twitch times-to-peak indicate that motor units are observably differentiated in the neonatal rabbit soleus. By inference, motor neurons thus innervate preferentially one or another fiber type, each having a characteristically different contraction time. Also supportive of the differentiation of motor neurons and the segregation of their innervation by fiber type is the correlation of peak motor unit twitch tension with contraction time such that slow motor unit tensions were on average smaller than those from fast motor units (Fig. 3). As will be argued in Chapter 3, fast and slow motor neurons differ not only in the physiology but also in the number of muscle fibers which they innervate.

In the neonatal cat soleus, Hammarberg & Kellerth (1975) also found differentiation into motor unit types based on a 2.7-fold range in motor unit twitch times-to-peak. The ages at which they examined the cat soleus corresponded to a time when that muscle is polyinnervated (Bagust, Lewis, & Westerman, 1973), so that selective innervation seems to characterize that system as well. The preliminary results from the rat soleus suggest that fibers in that muscle may also differentiate prior to the loss of polyinnervation and that, concomitant with fiber differentiation, motor units express distinct twitch physiologies, indicating a segregation of innervation.

The degree to which the innervation by any one motor neuron is segregated may vary considerably. Even at the extremes of the spectrum of motor unit twitch times-to-peak, motor units may still contain some mixture

of muscle fiber types. One would like to approach this issue directly and quantitatively with the glycogen-depletion method used to identify members of a motor unit in the adult (Edstrom & Kugelberg, 1968). Unfortunately, this method is not applicable to neonatal rabbit soleus. Attempts to use the method with neonatal preparations in vitro met with no success, presumably because the enzymic machinery for the breakdown of glycogen is not well differentiated (Dubowitz, 1963) and/or because the muscle is largely oxidative anyway.

Neonatal motor units which gave rise to intermediate twitch times-to-peak may have innervated a mixture of muscle fiber types. However, even these motor units may instead have innervated a single type of muscle fiber with intermediate twitch properties. Such a muscle fiber type might correspond to an intermediate-staining muscle fiber seen with ATPase histochemistry in the rabbit soleus around 14 days of age but not at 4 (Fig. 1b; Guth & Samaha, 1972; Kugelberg, 1973, 1976). Perhaps the physiological properties of these muscle fibers are differentiated before their ATPase-staining properties emerge, and hence they can be detected by the selective innervation by a specific motor neuron type. Such intermediate physiological properties are also seen in adult muscles where motor units contain exclusively one type of muscle fiber (Burke, 1981).

Physiological Differentiation of Muscle Fibers

Several groups have been concerned with the apparent contradiction between the high incidence of type II fibers and the slow whole muscle twitch properties observed in neonatal muscles (Karpati & Engel, 1967; Guth & Samaha, 1972; Gutmann, Melichna, & Syrovy, 1974; Rubinstein, Pepe, & Holtzer, 1977; Gauthier, Lowey, & Hobbs, 1978). Guth & Samaha (1972) have

found that despite the distinct histochemical differentiation of muscle fibers in neonatal rabbit hindlimb muscles, there is in fact very little actomyosin ATPase present. Instead, it seems that type II fibers contain an ATPase with very high alkaline stability. To what extent, then, are the neonatal rabbit soleus muscle fibers differentiated, and which twitch contraction times correspond to which histochemical profiles? While it does appear that primary fibers are associated with slow-like myosin components and secondary fibers with fast-like components as judged by immunohistochemistry (Rubinstein & Kelly, 1981; Whalen et al., 1981), the physiological differentiaton of neonatal fibers has not been emphasized in the literature. The data presented and reviewed here demonstrate that the muscle fibers are indeed physiologically differentiated at a time corresponding to histochemical differentiation. Chapter 3 will argue that fast twitch properties are associated with ATPase dark staining fibers and slow twitch properties with ATPase light staining fibers.

Interpretation of Undifferentiated Muscle Fibers

The absence of variability in motor unit twitch times-to-peak from neonatal rat and embryonic rabbit soleus indicates simply that muscle fibers in these developing muscles are not well differentiated into types, as is also reflected by the uniformity in their ATPase histochemistry. The muscle fibers may, nonetheless, be committed to future expression of a particular type as suggested by the preliminary results from rat soleus. There, physiological and histochemical differentiation seem to emerge together, possibly prior to the loss of polyinnervation. In both muscles, then, motor neurons may innervate distinct populations of muscle fibers committed to future differentiation into one or another fiber type.

Because of the immature state of development of muscle fiber types, such a segregation could not, however, be verified with the physiological assay.

Developmental Significance

How could specificity of neuromuscular connections have arisen during development at a time when the muscle is polyinnervated? One class of possibilities considers that the pattern of connectivity seen just after birth results from an originally specific pattern of innervation. First, there might be a chemoaffinity (Sperry, 1963) such that fast motor neurons bear surface receptors for fast muscle fiber markers, and similar situations would obtain for the other muscle fiber and motor neuron types. During development, motor axons would actively search for appropriate synaptic sites. This possibility, however, is difficult to reconcile with the lack of specificity for muscle fiber types during regeneration in mammals (Kugelberg, Edstrom, & Abbruzzese, 1970; Brooke, Williamson, & Kaiser, 1971). Second, motor axons might instead show affinity for other motor axons of like type and preferentially fasciculate together down to the same end plates (Van Essen, 1982). Through inductive factors (Gutmann, 1976) or activity patterns coordinated among the several like inputs, each muscle fiber would then acquire the type defined by its inputs. Why neonatal rabbit and rat solei should appear as well-mixed checkerboards would not be easily explained under this hypothesis. One might instead expect fasciculating axons to innervate clusters of muscle fibers. Third, there is evidence that muscle fibers are born in at least two waves of myogenesis in which the first fibers born become predominantly slow-twitch and the secondary fibers become initially fast-twitch (Wirsen & Larsson, 1964; Kelly & Zachs, 1969; Ashmore et al., 1972; Kelly & Schotland,

1972). Kelly and Rubinstein (1980) have suggested a well-choreographed timing in rat extensor digitorum longus and soleus muscles in which slow motor neurons would grow first into the muscles and extensively polyinnervate the initial group of myotubes. Then, in a second wave of innervation, fast motor neurons would grow into the muscles and find a largely separate population of fibers available for polyinnervation.

A second class of possibility considers that the original innervation is nonspecific, but that subsequent elimination is selective prior to the time of differentiation of muscle fiber types. Perhaps muscle fiber types are determined independent of their original innervation. Muscle fibers of a particular type might then cause early elimination of incompatible inputs. Another possibility is that the prevailing inductive influence of a collection of inputs to an embryonic end plate determines the muscle fiber type, and then subsequently, the incompatible inputs are preferentially eliminated.

I have argued that both motor neurons and muscle fibers are differentiated into distinguishable physiological types at birth in the rabbit soleus and that at a time when the muscle fibers are polyinnervated motor neurons of a particular type preferentially share end plates with other motor neurons of the same type. In the future, it may be possible to more directly investigate the developmental basis of this neuromuscular specificity with anatomical techniques.

Chapter 2

RELATION OF NEUROMUSCULAR SYNAPSE ELIMINATION TO SPINAL POSITION OF RABBIT AND RAT SOLEUS MOTOR NEURONS[1]

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Mammalian muscle provides an accessible model system for the detailed study of plasticity in the nervous system. The pattern of neural connections changes dramatically and rapidly in prenatal and early postnatal muscle. Early in development, each muscle fiber receives transient innervation from several motor neurons. Subsequent elimination of the redundant inputs then leads to the adult state of single innervation. In rats, cats, and rabbits the process of synapse elimination occurs in the first few postnatal weeks (Redfern, 1970; Bagust, Lewis, & Westerman, 1973; Brown, Jansen, & Van Essen, 1976; Bixby & Van Essen, 1979a). Why particular connections are retained during maturation while others are lost continues to be an intriguing problem in developmental neurobiology. Are, for instance, some motor neurons better able to retain synaptic sites in the muscle than others?

Miyata and Yoshioka (1980a, 1980b) have reported a large difference in the ability of rat soleus motor neurons to retain neuromuscular synapses through the period of synapse elimination. Their results suggested that motor neurons from spinal root L4 lose approximately three quarters of their synapses, whereas those from L5 undergo no net loss. Such a striking difference, if confirmed, would open new approaches to the understanding of competitive synaptic interactions at the neuromuscular junction. For example, one could compare the physiological properties of synapses from two populations of motor neurons, one of which undergoes synapse elimination while the other does not. O'Brien (1981) took such an approach in the rat soleus, where he compared the quantal contents of end plate potentials evoked by stimulation of the L4 and L5 spinal roots. At 5 days of age he found that the quantal contents from the L4 spinal root averaged only one half that from the L5 root. This was interpreted as evidence that synapses

from the L4 root are weaker prior to elimination and are eliminated preferentially.

Miyata and Yoshioka (1980b) used an indirect method to estimate the relative synaptic field sizes of motor neurons. By sampling end plate potentials in response to L4 or L5 stimulation, they estimated the relative contribution of each spinal root to the innervation of the soleus at different stages of development. However, the overall degree of innervation by a spinal root reflects not just the number of synapses per contributing motor axon but also the number of contributing motor axons in that root. In fact, there is considerable variability in the proportion of motor axons contributing to the soleus muscle via each spinal root in the rat. Some rat soleus muscles are innervated exclusively by L4 and others exclusively by L5 (Miyata & Yoshioka, 1980b) while most lie in a broad spectrum in between (our unpublished observations). In the rabbit we have observed the pattern of soleus innervation to be even more variable: in the extreme, some soleus muscles are innervated exclusively by spinal roots L7 and S1 and others exclusively by S2 and S3. Such variability undoubtedly stems from the frequent rostral/caudal displacement of motor columns relative to vertebral segments (Sherrington, 1892; Romanes, 1951). Therefore, in order to properly assess the relative synapse loss between spinal roots one needs to measure the number of synapses made by individual motor axons within each root, not just the overall number of synapses per root.

In the course of experiments on the postnatal development of motor units in the rabbit soleus, we had the opportunity to make direct comparisons between the twitch tensions produced by individual motor axons in different ventral roots. The assay of individual motor unit sizes as

measured by twitch tensions has two advantages for the purpose of the present study. First, it is independent of the variability in the distribution of axons between the roots. Second, it allows for a much more thorough sampling of the muscle than does intracellular recording of end plate potentials. In the rabbit we found a large-scale loss of synapses from soleus motor neurons arising from all spinal segments. In order to resolve whether there might be a major species difference between rabbits and rats, we reexamined the rat soleus muscle using the same assay. Again, there was no indication of a major rostral/caudal bias in susceptibility to synapse elimination. Possible explanations for the disagreement with previous studies are considered in the Discussion. Some of the observations on the rabbit soleus have been presented in preliminary form elsewhere (Gordon & Van Essen, 1981; Van Essen, 1982).

METHODS

Experiments were conducted on the soleus muscles of male and female New Zealand White rabbits and Sprague-Dawley rats aged from birth to 18 days postnatal. Motor unit twitch tensions were measured with a sensitive isometric force transducer. The transducer in early experiments was a piezoelectric bimorph, while in later experiments it was a piezo-resistive unit (Aksjeselskapet Mikro-Electronikk, Horten, Norway, model AE 875). Twitch tensions of individual motor units were determined by splitting the ventral roots from spinal segments L7 through S3 as appropriate in the rabbit and segments L4 and L5 in the rat.

A number of animals received their soleus innervation exclusively or almost exclusively from a single spinal root and were not included in this

analysis. All animals used in this study had at least three motor units from each of two spinal roots. In the rabbit only spinal roots S1 and S2 were compared, except in two animals where L7 and S1 were compared instead. In a few rabbits a third spinal root contributed an additional 1-4 motor units to the soleus. These extra motor units amounted to only 2% of all those encountered and for convenience were excluded from the data base. There was no indication that the discarded units differed significantly from the included units. On average 17 motor units were examined in each rat and 31 in each rabbit.

RESULTS

Motor unit twitch tensions were obtained in rabbit and rat soleus muscles in two age groups: from birth to 5 days of age and from 11 to 18 days of age. In both species, the first group involved muscles which were extensively polyinnervated while the second group consisted of muscles whose fibers had nearly all become singly innervated (Brown et al., 1976; Bixby & Van Essen, 1979a).

Postnatal Changes in the Rabbit

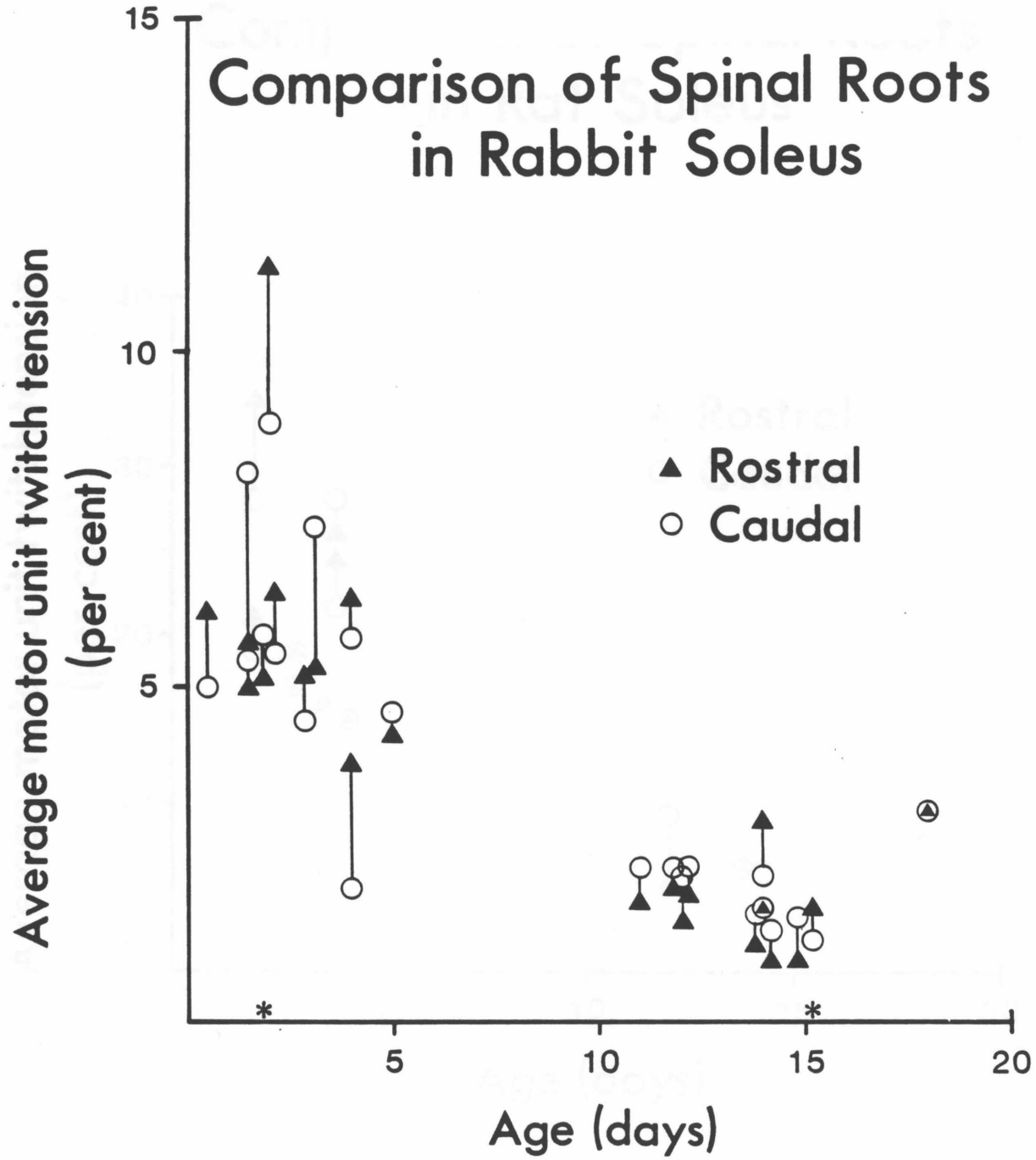
Figure 1 shows the average twitch tension, expressed as a percentage of the maximal direct tension, for motor units from the rostral and caudal spinal roots to rabbit soleus muscles of different ages. In the younger group (0-5 days), there was an insignificant difference in the average motor unit twitch tensions of the rostral (5.64 ± 0.24 [s.e.m.]%, n = 163) and caudal (5.94 ± 0.22 [s.e.m.]%, n = 194) contributing spinal roots.

Figure 1

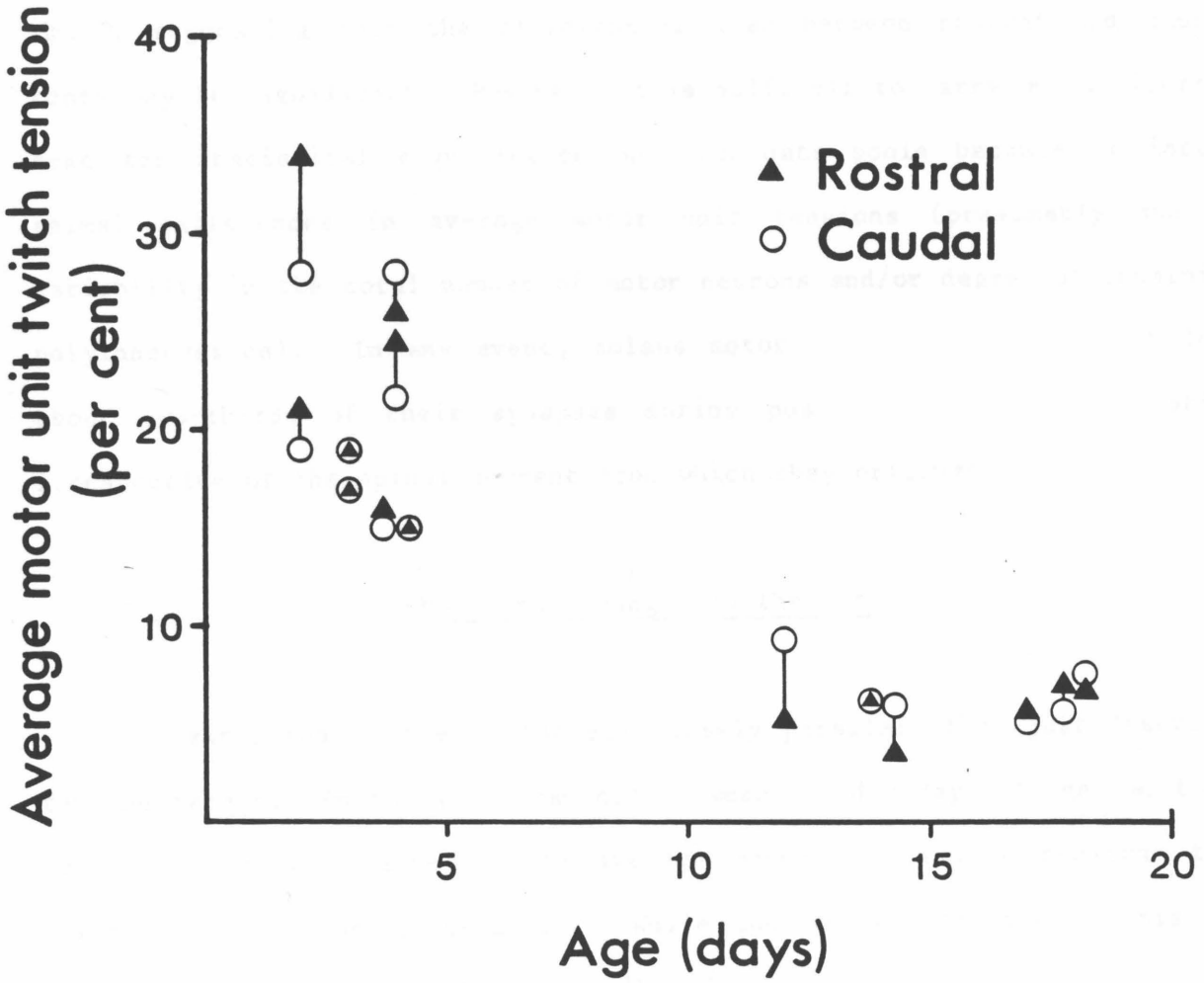
Innervation of rabbit soleus muscle by spinal roots L7, S1, and S2 during postnatal development. The average twitch tensions from both contributing spinal roots are plotted at various ages. When not overlapping, the average tensions for the two roots of a single animal are linked by a solid line. In all but two cases a filled triangle denotes the average motor unit twitch tension of the S1 root while an open circle denotes the average motor unit twitch tension of the S2 root. In the other two cases, designated by asterisks near the abscissa, a filled triangle corresponds to L7 and an open circle to S1.

Figure 2

Innervation of rat soleus muscle by spinal roots L4 and L5 during postnatal development. Filled triangles represent spinal root L4 while open circles represent the L5 root.


Comparison of Spinal Roots
in Rabbit Soleus
Average motor unit twitch tension
(per cent)
15
10
5
Rostral
Caudal
*
*
5
10
15
20
Age (days)

Comparison of Spinal Roots in Rat Soleus



This result suggests that spinal position per se plays no role in the ability of soleus motor neurons to form synapses during the initial period of neuromuscular innervation. By ages 11 to 18 days, motor neurons from both spinal roots had lost considerable numbers of synapses, with a hint that motor units from the rostral root (1.68 ± 0.12 (s.e.m.)%, n = 155) had declined in size slightly more than those from the caudal root (2.05 ± 0.13 (s.e.m.)%, n = 190). The standard errors for these measurements are quite small, suggesting that the differential loss between rostral and caudal roots may be significant. However, it is difficult to carry out a rigorous test for statistical significance on such data pools because of inter-animal differences in average motor unit tensions (presumably due to variability in the total number of motor neurons and/or degree of remaining polyinnervation). In any event, soleus motor neurons of the rabbit lose about two-thirds of their synapses during postnatal synapse elimination irrespective of the spinal segment from which they originate.

Postnatal Changes in the Rat

The situation in the rat soleus closely parallels that just described for the rabbit. In the rats examined between 2 and 5 days of age, we found no consistent difference in the average motor unit twitch tensions from spinal roots L4 and L5 (Fig. 2). While the overall average in this age group for L4 (23.0 ± 1.3 [s.e.m.]% of whole muscle tension, n = 59) slightly exceeded that for L5 (20.5 ± 1.2 [s.e.m.]%, n = 69), the difference was of marginal significance. By ages 12 to 18 days, the average motor unit twitch tensions in the animals examined had declined markedly and equally for both spinal roots (5.70 ± 0.62 [s.e.m.]% for L4

[n = 27]; 6.15 ± 0.39 [s.e.m.]% for L5 [n = 85]). By chance there happened to be a bias in five of the six older rats for a roughly fourfold greater number of motor neurons contributing from the L5 root. However, this did not affect analysis on a per motor neuron basis. Two conclusions are thus warranted. First, in contrast to the interpretation of Miyata & Yoshioka (1980b) we find clear evidence that synapses are lost in large numbers by soleus motor neurons of the rat passing through either contributing spinal root. Second, there appears to be no significant difference in the degree to which synapses are lost by either contributing spinal root in the rat soleus.

DISCUSSION

Results of this study support the hypothesis that spinal position per se plays at most only a minor role in the ability of a motor neuron to form or lose synapses during development. Because of limitations in our method we cannot rule out a subtle bias in the ability of spinal roots to compete for representation in muscle such as that reported by Wigston & Sanes (1982). First, twitch tensions of rabbit and rat soleus motor units vary over a large range in immature muscles (Gordon & Van Essen, 1981). Even with a large sample, it can be difficult to be sure whether differences of up to 20-30% in the means for different spinal roots reflect a genuine biological bias. Nonetheless, given a severalfold overall loss of synapses, any marked difference in the loss between spinal roots would have been obvious. Second, measurement of peak twitch tensions does not constitute a perfect assay of the number of muscle fibers in a motor unit. The measurements are subject to errors due to differences in fiber type, average fiber

cross-sectional area, and nonlinear summation of twitch tensions from individual muscle fibers within a motor unit. However, we found no striking evidence for a rostral/caudal segregation of motor unit types in either the rabbit or rat. Moreover, the simple architecture of the soleus tends to minimize nonlinear summation of twitch tensions (Lewis, Luck, & Knott, 1972). All in all, motor unit twitch tension should provide a reasonable assay of motor unit size and has the great advantage of providing large amounts of data from each experimental muscle.

The considerable biological variability in the distribution of soleus motor axons between spinal roots makes it highly advantageous to have a direct assay of motor unit size when comparing synapse elimination by spinal roots. It seems likely that the discrepancy between our findings and those of Miyata and Yoshioka (1980b) results from their use of a small sample with an assay which depends critically on the constancy of the pattern of innervation. In the eight animals which they examined at 6-11 days of age, they found that L4 innervation averaged 95 ± 6% (s.d.) of the muscle while L5 averaged 48 ± 15% (s.d.). In contrast, for the 15 animals aged 12 days through 1 month, they found that the relative innervation by L4 and L5 varied virtually at random, with both averaging about 50 ± 30% (s.d.). Miyata and Yoshioka argued that because the <u>average</u> degree of L4 innervation declined during synapse elimination while the <u>average</u> degree of L5 innervation remained the same, only L4 synapses were eliminated. Alternatively, a chance bias in their sample of young rats for a predominance of L4 motor axons and a more typical random distribution in the older sample could completely account for their data without invoking differential synapse loss between the two spinal roots. Indeed, our sample

of rat soleus muscles also contained a bias. In our case, however, it was the older group of rats, and the bias was for predominantly L5 innervation.

The differential efficacy of immature L4 and L5 synapses reported by O'Brien (1981) could be similarly accounted for by a small sample of animals with a large bias for spinal root L5. O'Brien's assay of quantal content on such a sample would have yielded much larger quantal contents on average for L5 than for L4 simply because of the greater contribution of L5 motor axons to the polyinnervation of each end plate. In the absence of estimates of the relative innervation between L4 and L5 in his sample, O'Brien's results are difficult to interpret. Miyata and Yoshioka (1980b) also sampled quantal contents, but they made the important restriction of sampling only end plates with single inputs from each of L4 and L5. In contrast with O'Brien, they found no statistically significant difference between quantal contents of the L4 and L5 inputs. Measures of average synaptic efficacy thus provide little support for the hypothesis that L4 synapses are smaller or weaker and therefore more likely to be eliminated during developmental synapse elimination.

Synapse elimination during neuromuscular development provides an opportunity to sort out and fine-tune neuromuscular connections. Whether this opportunity is used to advantage remains very much an open question. We have here reexamined the possibility that a motor neuron's ability to compete for synapses during development correlates with rostral/caudal position in the spine. Our data provide no support for such a hypothesis, nor do the results independently reported by Thompson (1982, 1983b) on rat soleus. However, many other factors may still play a role in determining which terminals win the competition for sole control of an end plate (see Purves & Lichtman, 1980; Grinnell & Herrera, 1981; Van Essen, 1982). Among

these factors are the pattern of neural activity, the extent of the parent axon's synaptic field, and possibly an intrinsic matching between particular motor neurons and muscle fibers.

Chapter 3

DEVELOPMENT OF MOTOR UNITS IN RABBIT SOLEUS MUSCLE

Introduction

The development of neuromuscular innervation serves as a model system for the more general problem of pattern formation in the nervous system. How are the phenomenal number of specific connections that characterize the nervous system generated? In mammalian muscle, the pattern of connectivity between motor neurons and muscle fibers begins in a plastic state and then undergoes at least one transformation to achieve an adult pattern of innervation. Typically, mammalian muscle fibers are polyinnervated severalfold around the time of birth and lose their redundant inputs over the ensuing few weeks (Redfern, 1970; Lichtman & Purves, 1980). The rationale behind this dramatic rearrangement has remained largely a matter for speculation.

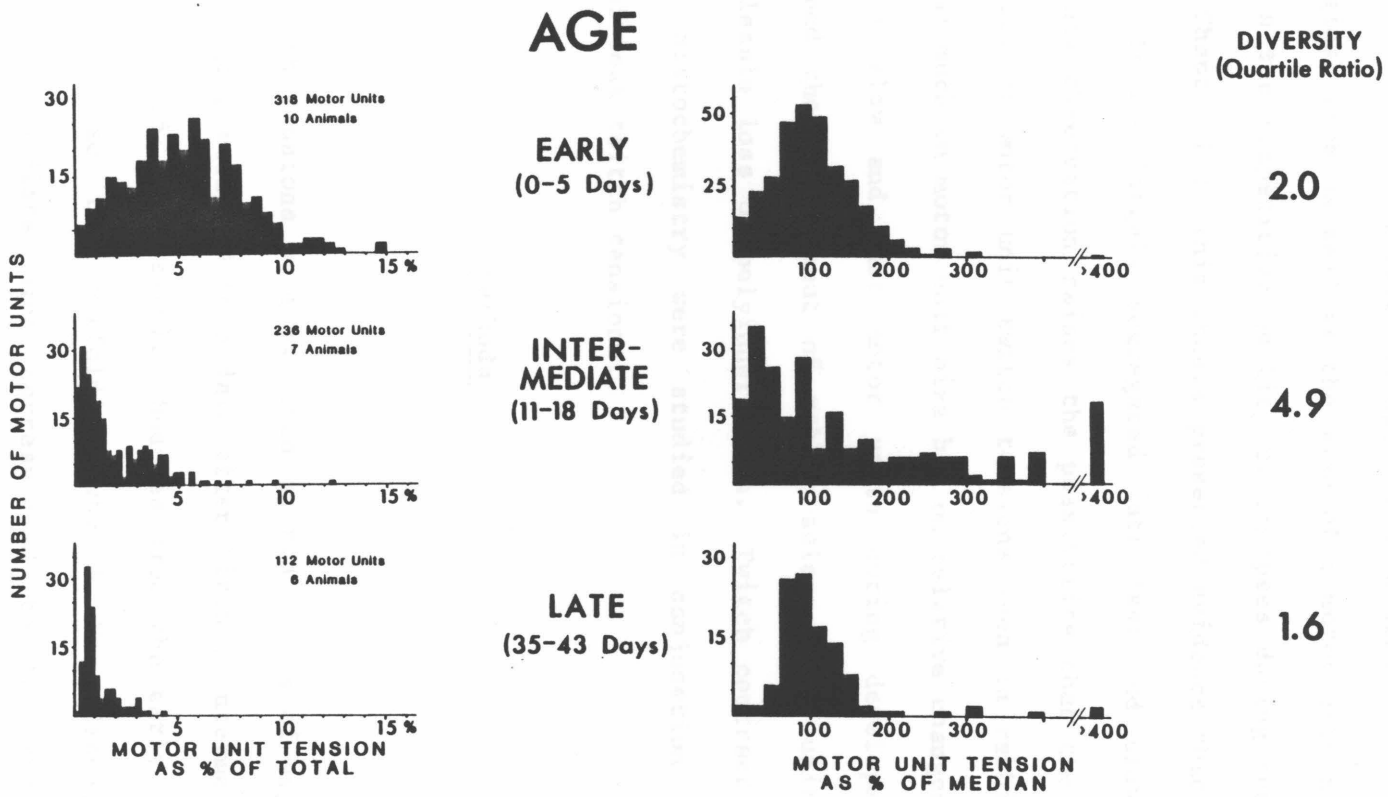
There is evidence from several systems that the distributions of motor unit sizes may undergo revealing changes both over the time period during which polyinnervation is lost and, as well, during the first few weeks of single innervation. In particular, Brown, Jansen, & Van Essen (1976) hypothesized on the basis of their limited data in rat soleus that motor neurons innervating large numbers of muscle fibers might lose relatively more synapses during the loss of polyinnervation than would motor neurons innervating a smaller number of muscle fibers. Motor neurons innervating many muscle fibers at birth were presumed to be overextended and less able to support their terminals in a competition with those from motor neurons with less extended aroborizations. This hypothesis predicts that the diversity of sizes within a population of motor units should decrease over the course of wholesale synapse elimination (Willshaw, 1981). While all motor units would decrease markedly in absolute size due to the loss of

polyinnervation, there would be relative convergence toward a mean as large motor units preferentially lost more synapses than small motor units.

In preliminary experiments, I and David Van Essen (1981) examined the distributions of a physiological measure of motor unit sizes in rabbit soleus muscle. By stimulating individual motor axons and recording the size of the resultant twitch tensions as a percentage of the total available twitch tension in the muscle we could estimate the number of muscle fibers belonging to each motor unit (Fig. 1). In agreement with the Brown et al. (1976) hypothesis, we found that between birth and 5 to 6 weeks of age, the diversity in motor unit twitch tensions in the rabbit soleus decreases. However, when we looked at twitch tensions shortly following the loss of polyinnervation, at 11 to 18 days of age, we found a remarkable increase in diversity, with some extremely small motor unit twitch tensions and some as much as 100 times larger. On the face of it, some originally very large motor units had apparently lost few synapses while other units had lost a disproportionate number. At 5 to 6 weeks of age, fewer of the very small and very large motor unit twitch tensions were found. In cat soleus, Westerman et al. (1973) likewise found a transient appearance of extremely small motor units late in the period of polyinnervation. These units were not seen in older muscles. Furthermore, the overall diversity in size of motor unit twitch tensions decreased from 2 to 6 weeks of age, to account for which a secondary synaptic reorganization was suggested (Bagust, Lewis, & Westerman, 1974). Such a reorganization would allow the overly small motor units to recoup some of their lost territory at the expense of the largest motor units. In the rat soleus, Brown et al. (1976) also noted that the smallest motor unit twitch tensions in a 6-week-old animal were larger than the smallest seen at 15 and 17 days

Figure 1

Motor unit peak twitch tensions from the rabbit soleus at three postnatal ages. The three histograms on the left depict data pooled without scaling and expressed as percent of whole muscle twitch tension. Changes in mean twitch tension between the early pool and the later two pools reflect loss of polyinnervation. The three histograms on the right depict the same data scaled relative to the median of each animal and then pooled. The resulting histograms better reflect the distribution from any particular animal at a given age. The width or "diversity" of each pool of motor unit sizes is measured as the ratio of upper to lower quartiles. Note that the diversity increases between the early and intermediate ages and then decreases by the late age (Figure previously described in Gordon & Van Essen, 1981).


AGE
DIVERSITY
(Quartile Ratio)
318 Motor Units
10 Animals
236 Motor Units
7 Animals
112 Motor Units
6 Animals
EARLY
(0-5 Days)
INTER-
MEDIATE
(11-18 Days)
LATE
(35-43 Days)
2.0
4.9
1.6
NUMBER OF MOTOR UNITS
MOTOR UNIT TENSION
AS % OF TOTAL
MOTOR UNIT TENSION
AS % OF MEDIAN

of age, just after the loss of polyinnervation in that species. They independently suggested the possibility of a secondary synaptic reorganization following the period of wholesale synapse elimination.

The physiological type as well as the size of a motor unit may play a significant role in the competitive ability of synapses during the loss of polyinnervation. Chapter 1 of this thesis presented evidence that at birth motor units were already largely segregated into fast and slow physiological types. This observation raises the possibility that the dramatic changes in diversity of motor unit twitch tensions seen in rabbit soleus may be based not so much on motor unit size but on relative changes between twitch tensions of slow and fast motor units during development. I therefore reexamined the development of rabbit soleus motor units during and after the wholesale loss of polyinnervation. Twitch contraction times and muscle fiber histochemistry were studied in conjunction with the previous measure of peak twitch tensions.

Methods

Physiology

Motor unit twitch tensions were examined in two series of experiments at three postnatal ages, early (0 to 5 days after birth), intermediate (11 to 18 days), and late (35 to 43 days). Muscles from the early age group were polyinnervated three to fourfold (Bixby & Van Essen, 1979a; Chapter 2). The intermediate ages corresponded to the period just following the developmental loss of polyinnervation while the late period corresponded to a young adult state of affairs. The first series of experiments, a preliminary investigation, used a piezoelectric bimorph to

examine the peak twitch tensions of individual motor units as recorded on a storage oscilloscope. In the second series, motor unit twitch tensions were measured with a piezoresistive force transducer and recorded through an analog to digital converter on a computer. The temporally more accurate transducer in conjunction with an improved medium for data storage made possible the simultaneous analysis of both peak twitch tension and time to peak twitch tension. (Details may be found in the Methods chapter.) Motor unit twitch tensions were also examined in the rat soleus at corresponding ages with the second paradigm.

Partial denervations of the rabbit soleus muscle were performed by section of the contribution to the sciatic nerve from spinal root S1. Seven muscles which had been partially denervated shortly after birth (0-4 days) were used for physiological recordings of motor unit twitch tensions at 11 to 16 days of age. Controls for equality of direct muscle and nerve-elicited twitch tensions obviously could not be performed, although the direct twitch tension, as usual, was not allowed to change by more than 15% over the course of an experiment.

Intracellular recordings of end plate potentials were conducted on seven animals aged from 14 to 32 days of age in an attempt to locate polyinnervated end plates during a period of possible secondary reorganization of synapses following the postnatal loss of polyinnervation. Methods were those of Bixby and Van Essen (1979a).

Anatomy

The frequencies and cross-sectional areas of various muscle fiber types were examined through development with a histochemical stain for actomyosin ATPase (Guth & Samaha, 1970; see Methods chapter for details).

The most consistent results were obtained by preincubating muscle cross sections at pH 10.4 prior to incubation with ATP (see Methods chapter), and all results in this chapter are based upon this technique. Light staining profiles thus corresponded to type I, presumably slow twitch fibers, and dark staining profiles corresponded to type II, presumably fast twitch fibers. (See Methods chapter for details.) Two soleus muscles were examined quantitatively and three or four qualitatively for each age group. Cross sections through the midbelly of each muscle were divided into three regions, ventral, interior, and dorsal, within which samples of fiber frequency and 50 cross-sectional areas for each fiber type were made. Whole muscle averages were then constructed by appropriately weighting contributions from each of the three regions. Frequency and size analyses were performed on two muscles aged 3 days old (#347 and #374), two muscles aged 13 and 15 days old respectively (#350 and #352), two muscles aged 36 days old (#386R and #388L), one muscle from an adult mother rabbit (#389), one muscle aged 13.5 days old which had been denervated at birth (#327), and one muscle aged 15 days old which had been partially denervated at birth (#337A).

Silver stains of terminal processes were performed throughout development as described in the Methods chapter. Particular attention was paid to the period during and after the intermediate age in hopes of demonstrating an anatomical correlate of a possible secondary reorganization of synapses.

Results

Physiology

When motor unit twitches were examined in a preliminary study of peak tension in three postnatal age groups, several dramatic changes with development were found (Fig. 1; Gordon & Van Essen, 1981). As expected from the loss of polyinnervation between the early and intermediate age groups, motor unit twitch tensions as a percent of total available twitch tension in the muscle declined. However, the relative diversity in twitch tension between motor units increased dramatically following the loss of polyinnervation. Diversity was measured independent of the size of the distribution as the ratio of the upper quartile to the lower quartile. Other size-independent measures were also calculated (e.g., the ratio of the standard deviation to the mean), but the quartile ratio seemed the most robust, gave a quasi-linear measure of a distribution's breadth, and avoided parametric statistics.

If twitch tensions were a good measure of the number of muscle fibers innervated by a single motor neuron, then the increase in diversity by the intermediate age could be interpreted as a preferential loss of synapses by a subset of motor neurons. In absolute terms, some motor unit twitches following the loss of polyinnervation were as large (expressed as a percentage of total tension) as some of the largest twitches in the neonate. Other motor units gave extemely small twitches, being only 0.04% of the total twitch strength available. The simplest explanation for this result was that small motor units preferentially lost synapses during the course of synapse elimination while large motor units preferentially retained synapses. It was if the same motor units which were less able to

form synapses initially were also less able to retain them during synapse elimination.

An examination of motor unit twitch strengths at a later age, 35 to 43 days after birth, yielded a provocative result. The distribution of motor unit twitch tensions was markedly less diverse than it had been in either of the two younger age groups. An obvious possibility was that there had been a synaptic reorganization within the muscle in which small motor units had acquired more synaptic sites at the expense of large motor units. How such a reorganization could occur in a muscle presumed to be singly innervated was an intriguing puzzle.

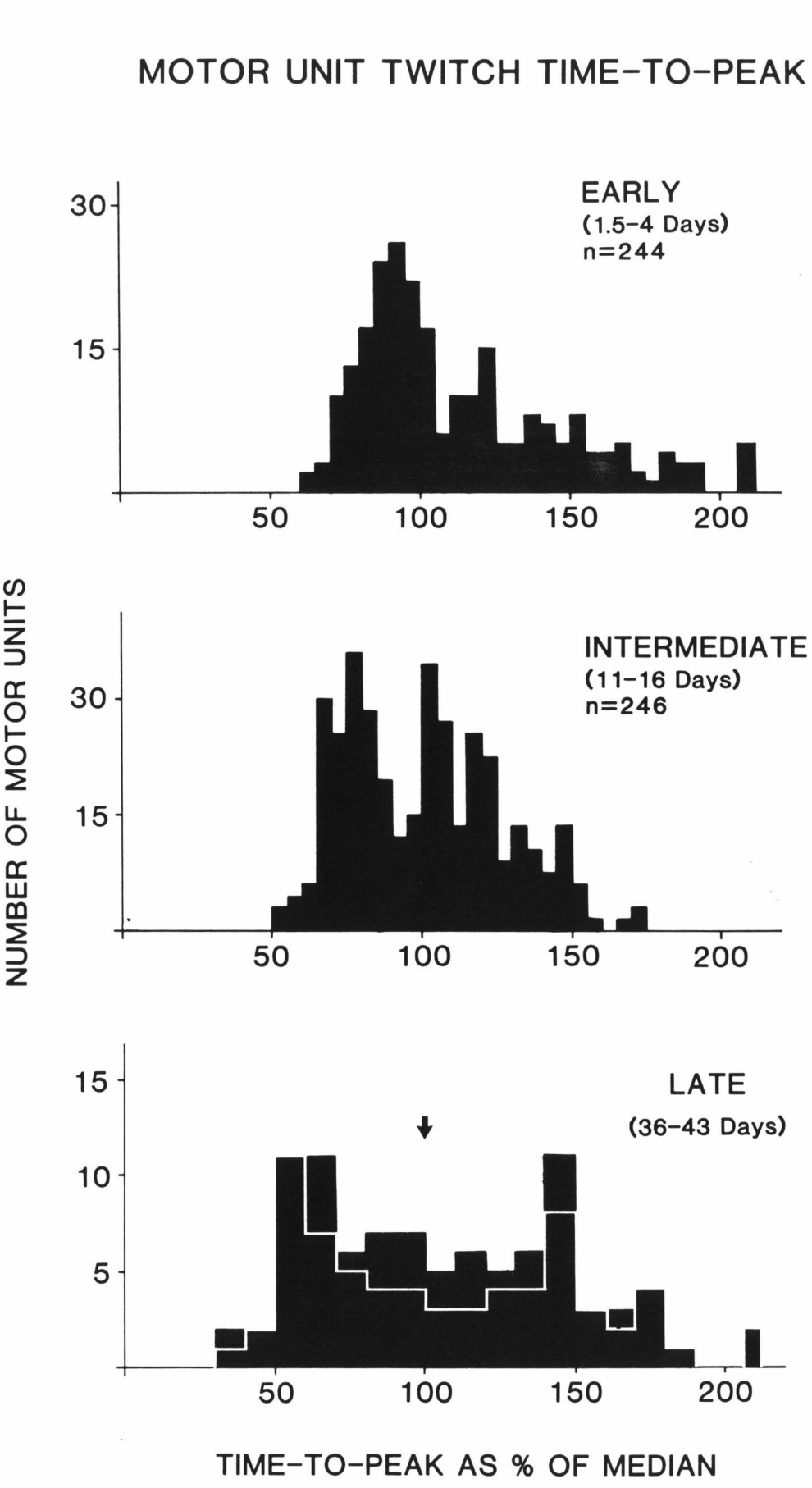
Comparative Development of Slow and Fast Motor Units

The results in Chapter 1 strongly suggest that motor units of the neonatal rabbit soleus are already well segregated by speed of contraction even in the neonate and despite considerable polyinnervation. It then became a likely possibility that the changes in diversity of motor unit twitch tensions described above could represent relative changes between the twitch sizes of slow and fast motor unit twitch tensions. In this light, peak motor unit twitch tensions were reexamined with respect to twitch time-to-peak in the three developmental ages described above.

Motor units were classified as slow or fast twitch according to their relative position within a pool of motor unit contraction speeds for each age group (Fig. 2). Because the average motor unit twitch time-to-peak differed between animals, the temporal data were scaled relative to the median of each animal prior to pooling. Differences in temperature of up to 2 C° between experiments may have contributed to this variability, but there seems to have been a major component of individual variability as

Figure 2

Motor unit twitch times-to-peak from the rabbit soleus muscle at three developmental ages. Twitch tensions from individual motor units were analyzed for time-to-peak tension and tension at peak. Prior to pooling, the temporal data were scaled relative to the median twitch time-to-peak of each animal. Three of the four animals pooled in the late age group gave a good segregation of slow and fast twitches, and the histogram of their motor units is shown outlined in white. Motor units were split into two populations of slow and fast as indicated by an arrow over each histogram. (Note that the data presented in this and the following four figures come from a distinct series of experiments from that presented in Fig. 1. See the data summary table in the Methods chapter.)


MOTOR UNIT TWITCH TIME-TO-PEAK
EARLY
(1.5-4 Days)
n=244
INTERMEDIATE
(11-16 Days)
n=246
LATE
(36-43 Days)
NUMBER OF MOTOR UNITS
TIME-TO-PEAK AS % OF MEDIAN
30
15
10
5
50
100
150
200

well. In particular, whole muscle contraction times were reported to vary as much as twofold in adult muscles studied *in vivo* (Bagust, 1972) and varied by somewhat less in the present *in vitro* study (50% range among the nine animals in the early age group [377 ± 57 (s.d.) ms], 40% range among the eight animals in the intermediate age group [247 ± 32 ms], and 10% range among the four animals in the late age group [332 ± 54 ms]). The resulting pools of scaled data better reflect the distribution of twitch times-to-peak from any given animal than do unscaled pools. For purposes of a comparative analysis between slow and fast motor units, motor units were then split into two populations based upon their relative position within the overall distribution of times-to-peak at a given age. At early and intermediate ages, the distributions of times-to-peak were easily split in two because of their bimodal nature. The late distribution of data pooled from four animals was not obviously bimodal. However, the distribution from three of the four animals was bimodal, and the overall distribution was split accordingly. Furthermore, the chosen split point (indicated by an arrow above the histogram in Fig. 2) divided the population of motor units into groups with distinctly different average motor unit peak twitch tensions.

Motor unit peak twitch tensions were pooled separately according to whether their twitch times-to-peak classified them as slow or fast as determined above. In each of the three developmental ages examined, the median slow motor unit twitch tension was found to be smaller than that of fast motor units (Fig. 3). The differences in motor unit twitch sizes reflected either differences in the number of muscle fibers innervated or in the average tensions elicited by the muscle fibers belonging to each motor unit.

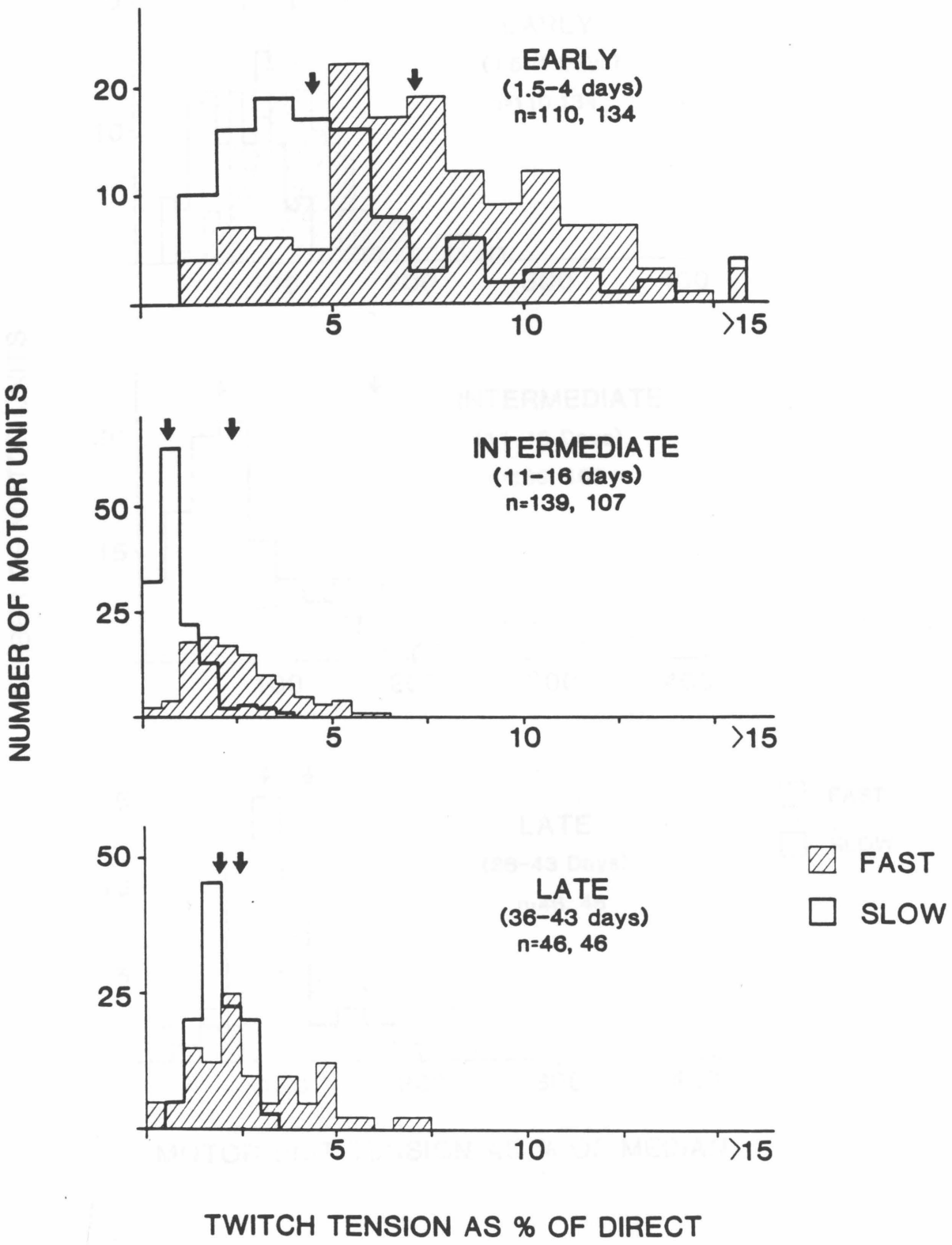
Figure 3

Slow and fast motor unit twitch tensions as % of direct. Motor units in three age groups were divided into pools with slow and fast twitch properties according to the splits described in Fig. 2. The resulting distributions of peak twitch tensions for slow and fast motor units are plotted here for three age groups. Arrows indicate the median for each distribution (early: slow = 4.5%, fast = 7.3%; intermediate: slow = 0.75%, fast = 2.4%; late: slow = 1.8%, fast = 2.4%). Number of slow motor units are listed prior to number of fast motor units.

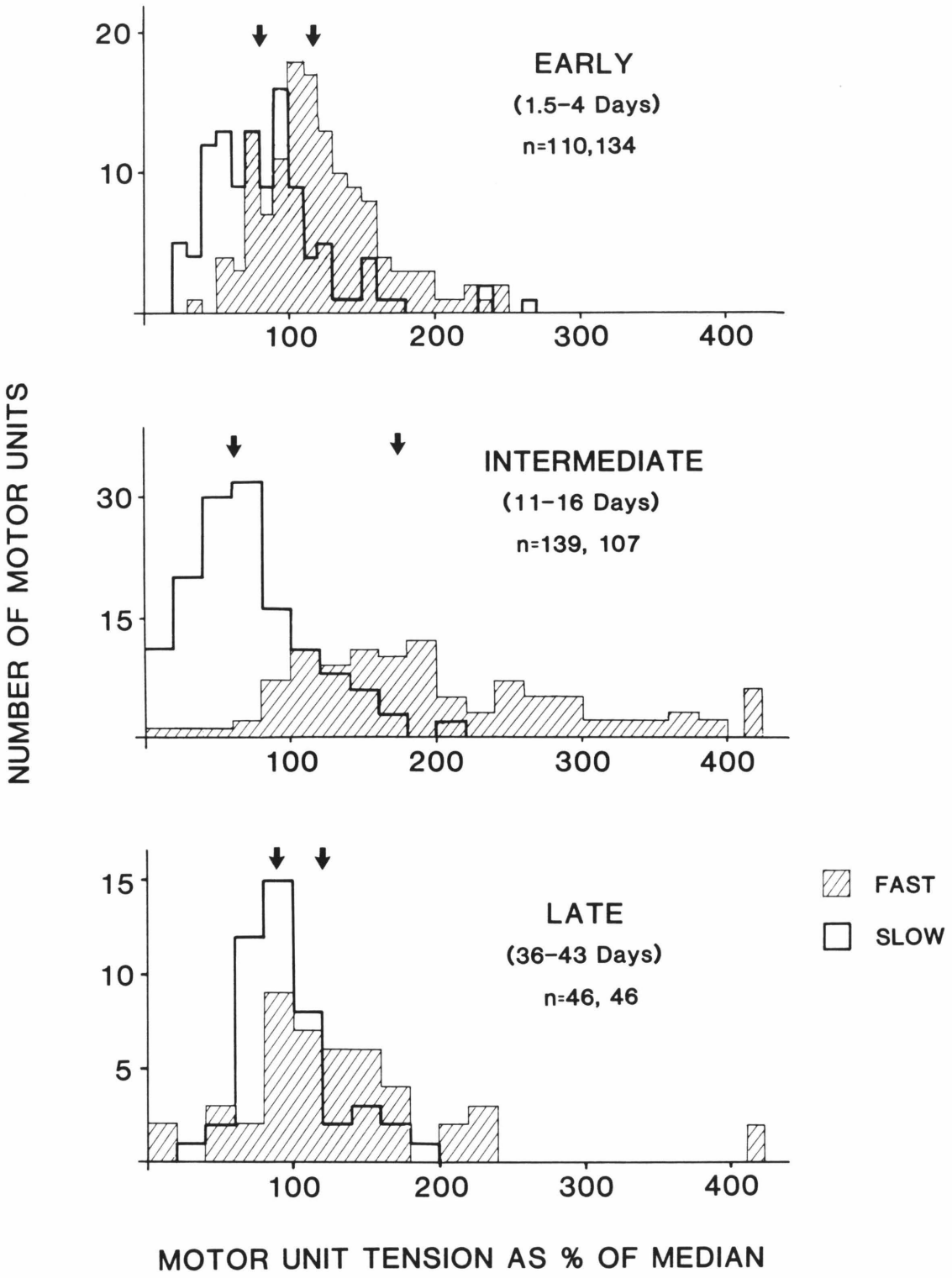
Figure 4

Slow and fast motor unit twitch tensions as % of median. Same data as in Fig. 3, but twitch tensions were scaled relative to the overall median for each animal and then separately pooled as fast or slow motor units according to the splits described in Fig. 2. Note in particular that the twitch tensions of fast motor units become relatively larger than those of slow motor units with the loss of polyinnervation between the early and intermediate ages. Arrows indicate the median for each distibution (early: slow = 79%, fast = 117%; intermediate: slow = 63%, fast = 179%; late: slow = 95%, fast = 134%).

SLOW AND FAST MOTOR UNIT TWITCH TENSIONS



RELATIVE SLOW & FAST MOTOR UNIT TWITCH TENSIONS



The relative sizes of average slow and fast motor unit twitch tensions were examined by scaling the motor unit twitch data to the median for all motor units from each animal and then separately pooling the slow and fast motor units (Fig. 4). The relative sizes changed twice during development. The median twitch tensions for the fast and slow units differed by a factor of 1.48 in the early group, by 2.84 in the intermediate group, and by 1.41 in the late group. Thus, much of the overall diversity in size of motor unit twitches seen in the preliminary experiments at the intermediate age (Fig. 1) can be accounted for by a relative diversification between slow and fast motor unit twitch strengths at that age.

It was possible to analyze the diversity of motor unit twitch sizes within separate populations of slow or fast motor units. Motor units were split into slow or fast populations and then scaled to the median of each population from individual animals prior to pooling over all animals at a given age (Figs. 5 and 6). For both slow and fast units there was a small but consistent increase in diversity of motor unit twitch sizes at the intermediate age which was followed by a decrease in diversity at the late age. The greatest diversity was found among slow motor units in the intermediate age group and was associated with the appearance of some very small and slow motor unit twitch tensions. When motor units of the rat soleus were scaled to the median twitch size on a per animal basis and without regard to contraction speeds, very similar distributions to those of either slow or fast motor units of the rabbit soleus were found (Fig. 7).

To examine whether the diversification of motor unit twitch sizes after the loss of polyinnervation was the result of a competition between motor units, rabbit soleus muscles were partially denervated between birth and 4 days of age and examined at 11 to 16 days of age.[1] The results were

Figure 5

Changes in diversity of slow motor unit twitch tensions in rabbit soleus. Same data as in Fig. 3, but twitch tensions from slow motor units were scaled relative to their median on a per animal basis and then pooled. The relative diversity of slow motor units can thus be compared at each of three developmental ages.

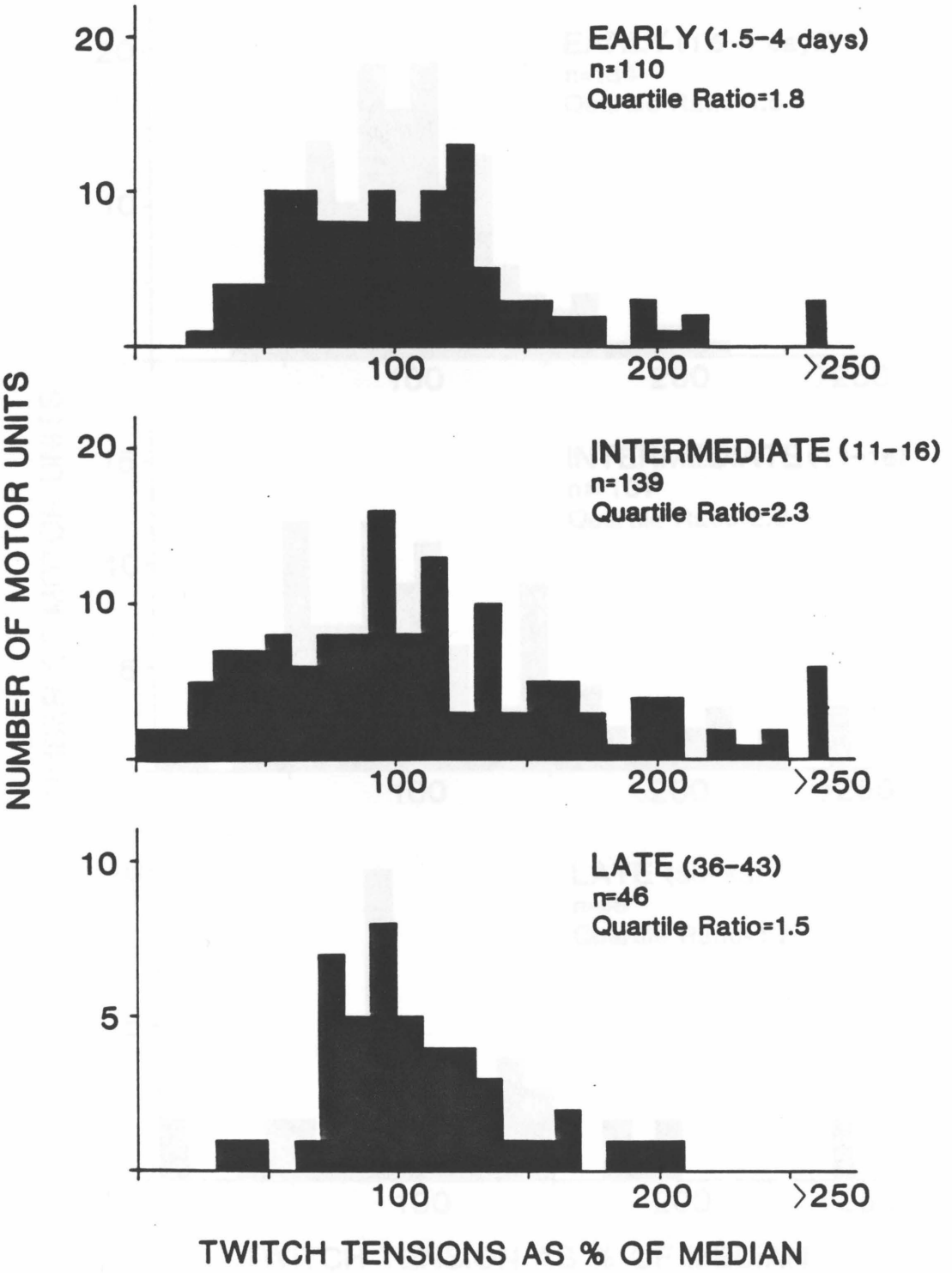
Figure 6

Changes in diversity of fast motor unit twitch tensions in rabbit soleus. Same data as in Fig. 3, but twitch tensions from fast motor units were scaled relative to their median on a per animal basis and then pooled. The relative diversity of fast motor units can thus be compared at each of three developmental ages.

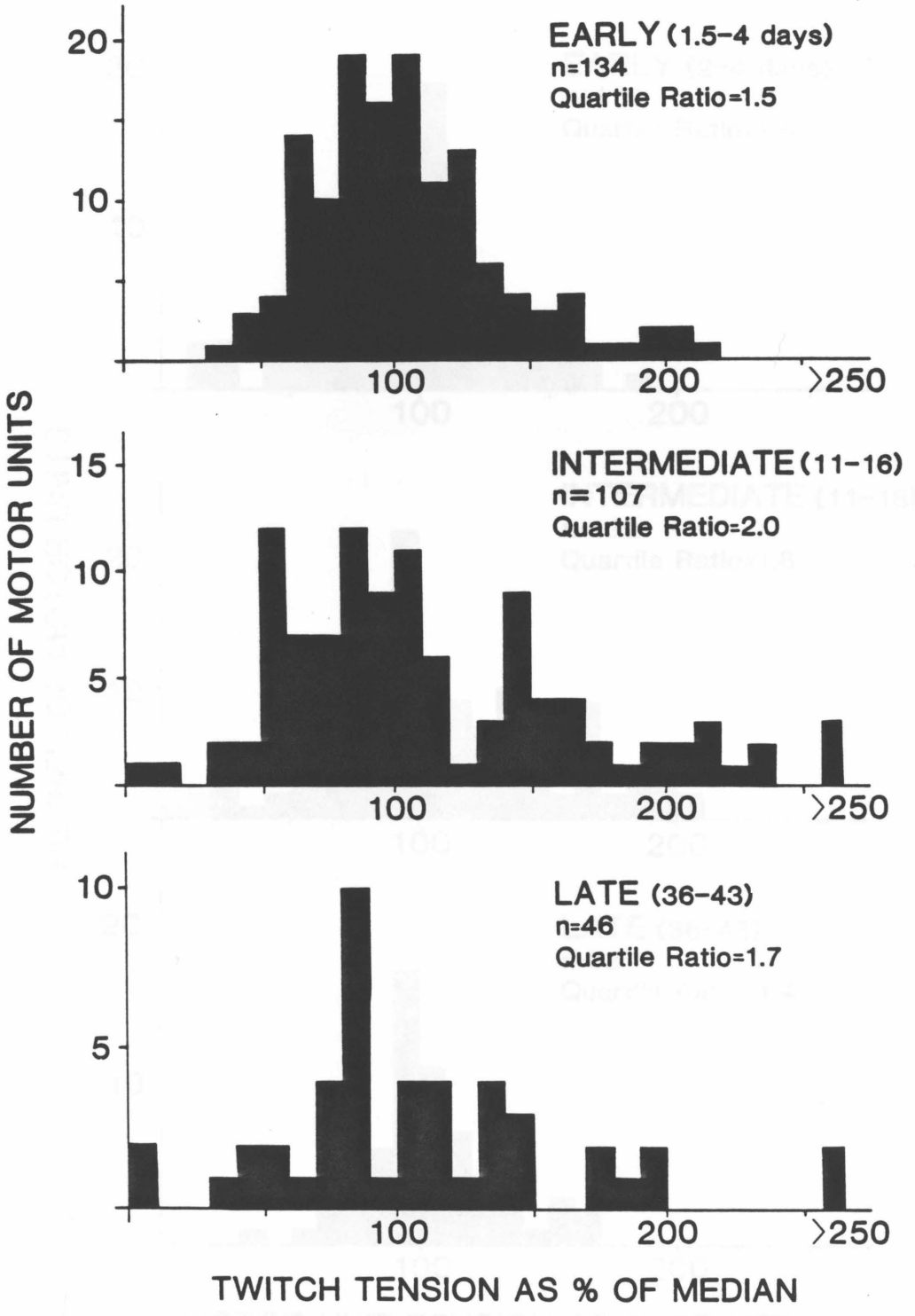
Figure 7

Changes in diversity of motor unit twitch tensions in rat soleus. As in Figs. 5 and 6, but here depicting pools of all motor unit twitch tensions from the rat soleus without regard to contractile properties. Data of Brown et al. (1976) when similarly pooled and analyzed yielded very similar quartile ratios, being 1.6 at the early age, 2.0 at the intermediate age, and 1.7 at the late age.

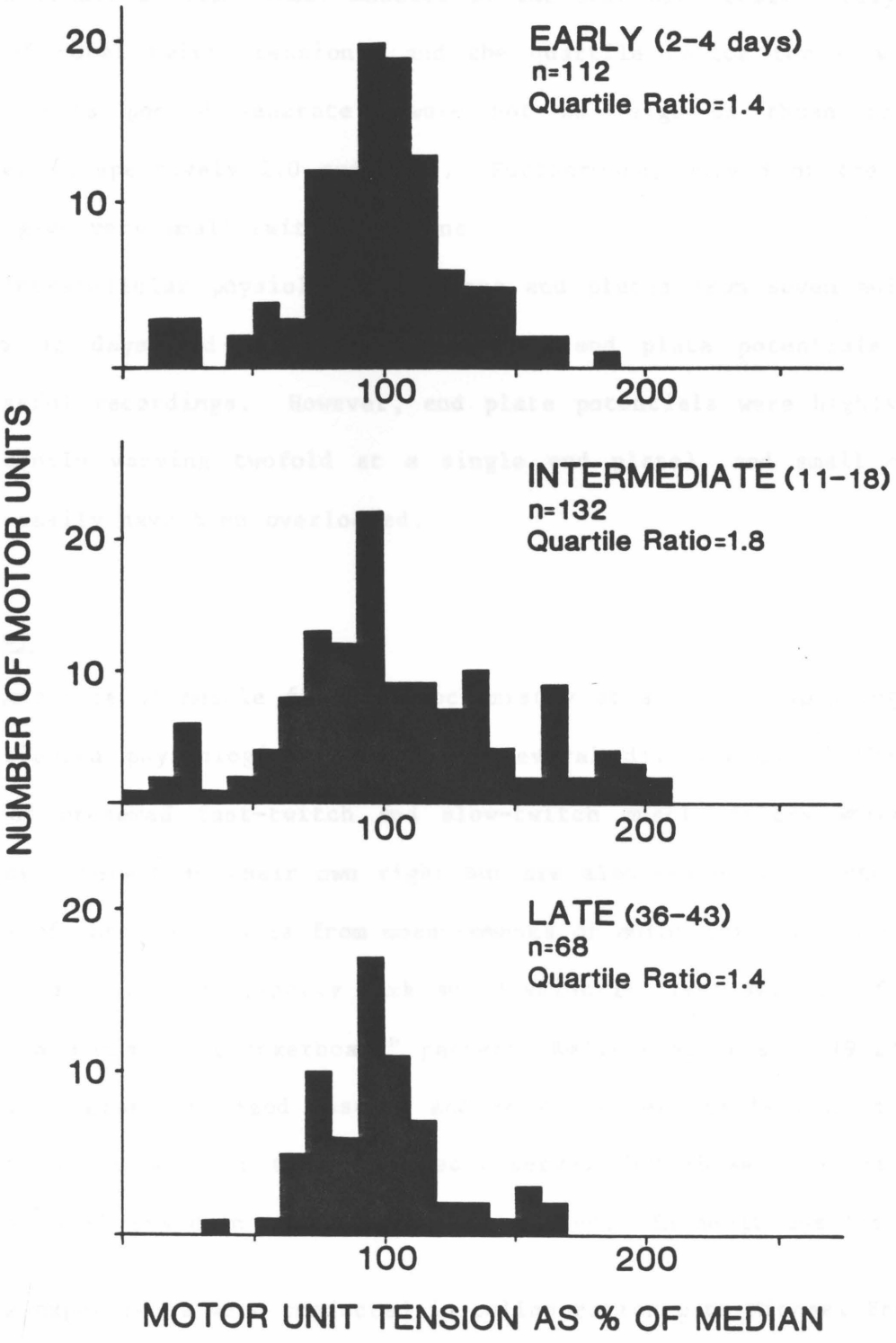
DIVERSITY IN TWITCH TENSIONS FOR SLOW MOTOR UNITS OF RABBIT SOLEUS



DIVERSITY IN TWITCH TENSIONS FOR FAST MOTOR UNITS OF RABBIT SOLEUS



DIVERSITY IN TWITCH TENSIONS FOR MOTOR UNITS OF RAT SOLEUS



that all the trends between early and intermediate aged normal soleus muslces were also found between normal early muscles and partially denervated intermediate muscles but to a lesser extent. The average twitch tension of both slow and fast motor units was larger than the corresponding twitch tensions from normal muscles of the same age (respectively 3.0% and 5.9% of total twitch tension), and the quartile ratios for slow and fast motor units pooled separately were not as large as those from normal muscles (respectively 2.0 and 1.7). Furthermore, only 3 of the 109 motor units gave very small twitch tensions.

Intracellular physiology of soleus end plates from seven animals aged 14 to 32 days old revealed no compound end plate potentials from 122 successful recordings. However, end plate potentials were highly variable (frequently varying twofold at a single end plate), and small components could easily have been overlooked.

Anatomy

Analysis of muscle fiber histochemistry at ages corresponding to those investigated physiologically revealed several differences in the development of presumed fast-twitch and slow-twitch muscle fibers which are not only of interest in their own right but are also essential in the interpretation of motor unit size from measurements of motor unit twitch tensions.

At all ages, distinctly dark and distinctly light staining fibers were found in a mosaic "checkerboard" pattern (Kelly & Schotland, 1972). In one of the intermediate aged muscles and in all older muscles, a third intermediate staining fiber type was also observed, but these never made up more than a small fraction of the total population. In adult muscles, the dark

[1]These experiments were conducted in collaboration with Michael Brown.

staining fibers are defined as type II and have been interpreted as fast twitch, and the light staining are defined as type I and have been interpreted as slow twitch (Brooke & Kaiser, 1970; Burke, 1981). Intermediate staining fibers are probably type IIC as they stain darkly when preincubated at pH 4.10 (Fig. 3 of the Methods chapter; Brooke & Kaiser, 1970; Edstrom, 1976).

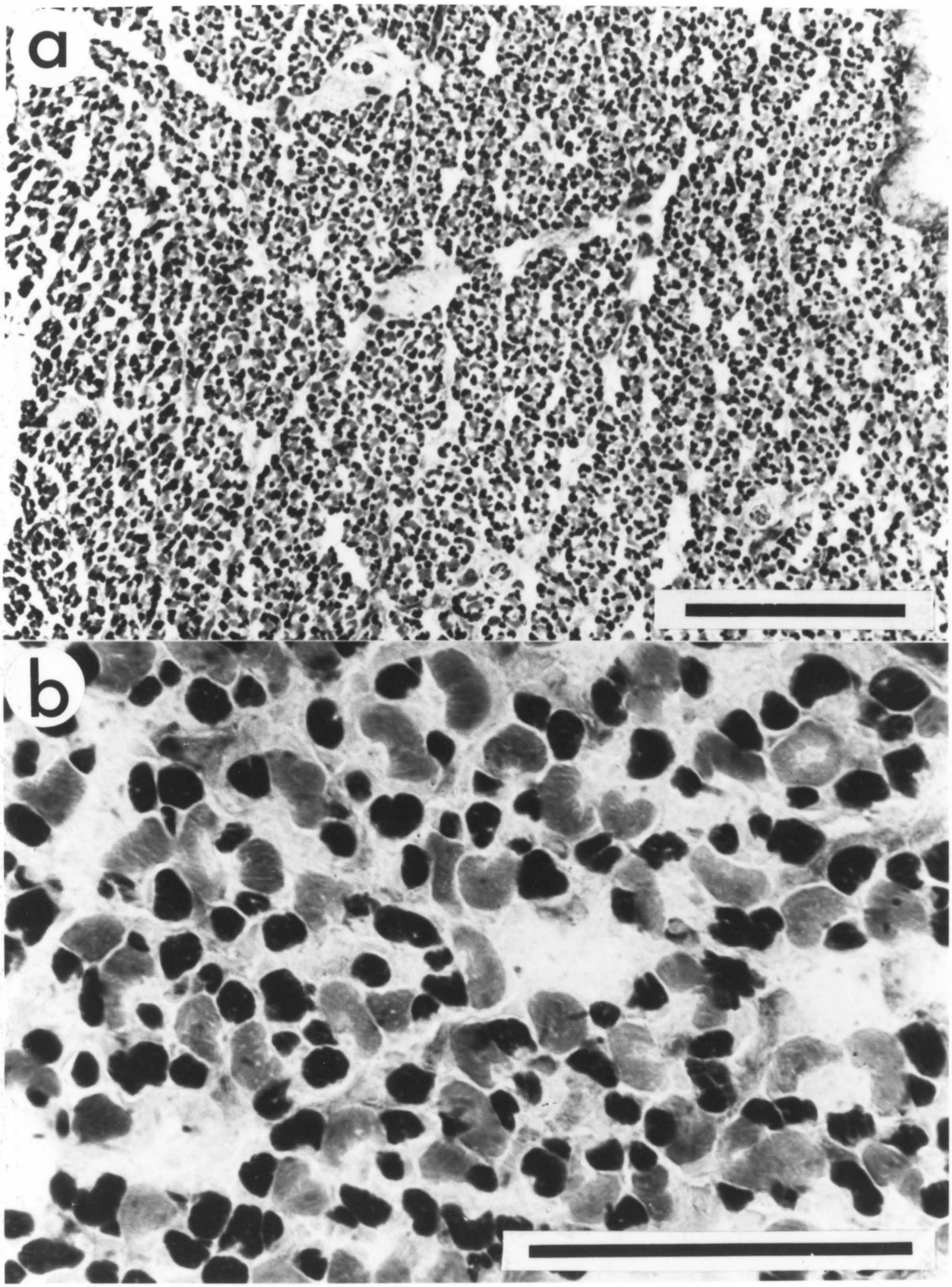
In muscles from the early age group (Fig. 8), many fibers were still in the myotube stage as defined by their centrally located nuclei (Engel & Karpati, 1968; Guth & Samaha, 1972). Nonetheless, fibers were distinctly stained either light or dark. Type I fibers were larger (145 ± 42 [s.d.] μm^2 on average across two muscles) than type II fibers (66 ± 32 μm^2), reflecting their probable origin as primary myotubes (Wirsen & Larsson, 1964; Kelly & Zachs, 1969; Ashmore et al., 1972; Kelly & Schotland, 1972). Fiber areas were more variable in the youngest muscles and in particular were most variable for type II fibers. Presumably, those myotubes which had been formed most recently accounted for the smallest type II fibers and hence for the increased variability in that fiber type. Type I fibers accounted for 31% of the fibers (ranging from 22 to 45% depending on region and muscle) and type II fibers for 69% in the two muscles examined at this age. Neither fiber type was significantly larger in one region of a muscle than another. One of the muscles (illustrated in Fig. 8) contained more frequent myotubes, and the average fiber size in this muscle was about 15% smaller than that of the other muscle. Though the two muscles were both from 3-day-old animals they were apparently at slightly different developmental stages. Both fiber types were evenly distributed across the developmentally less mature muscle, but there was a slight gradient across the other muscle such that type I fibers were

Figure 8

Three-day-old rabbit soleus stained for ATPase following preincubation at pH 10.4 (animal #347). (a) Cross section through midbelly of muscle. (Scale bar = 300 μm.) (b) Representative enlargement (Scale bar = 100 μm.) Lighter staining, larger profiles correspond to primary myotubes which are though to be slow twitch. Dark staining, small profiles correspond to secondary myotubes which are thought to be fast twitch.

Figure 9

Fifteen-day-old rabbit soleus stained for ATPase following preincubation at pH 10.4 (animal #352). (a) Cross section through midbelly of muscle. Dorsal margin appears at top of figure. (Scale bar = 500 μm.) (b) Representative enlargement depicting three muscle fiber types. Light staining profiles are type I muscle fibers, dark staining profiles are type II muscle fibers, and intermediate staining profiles are a subclass of type II, probably type IIC. (Scale bar = 100 μm.)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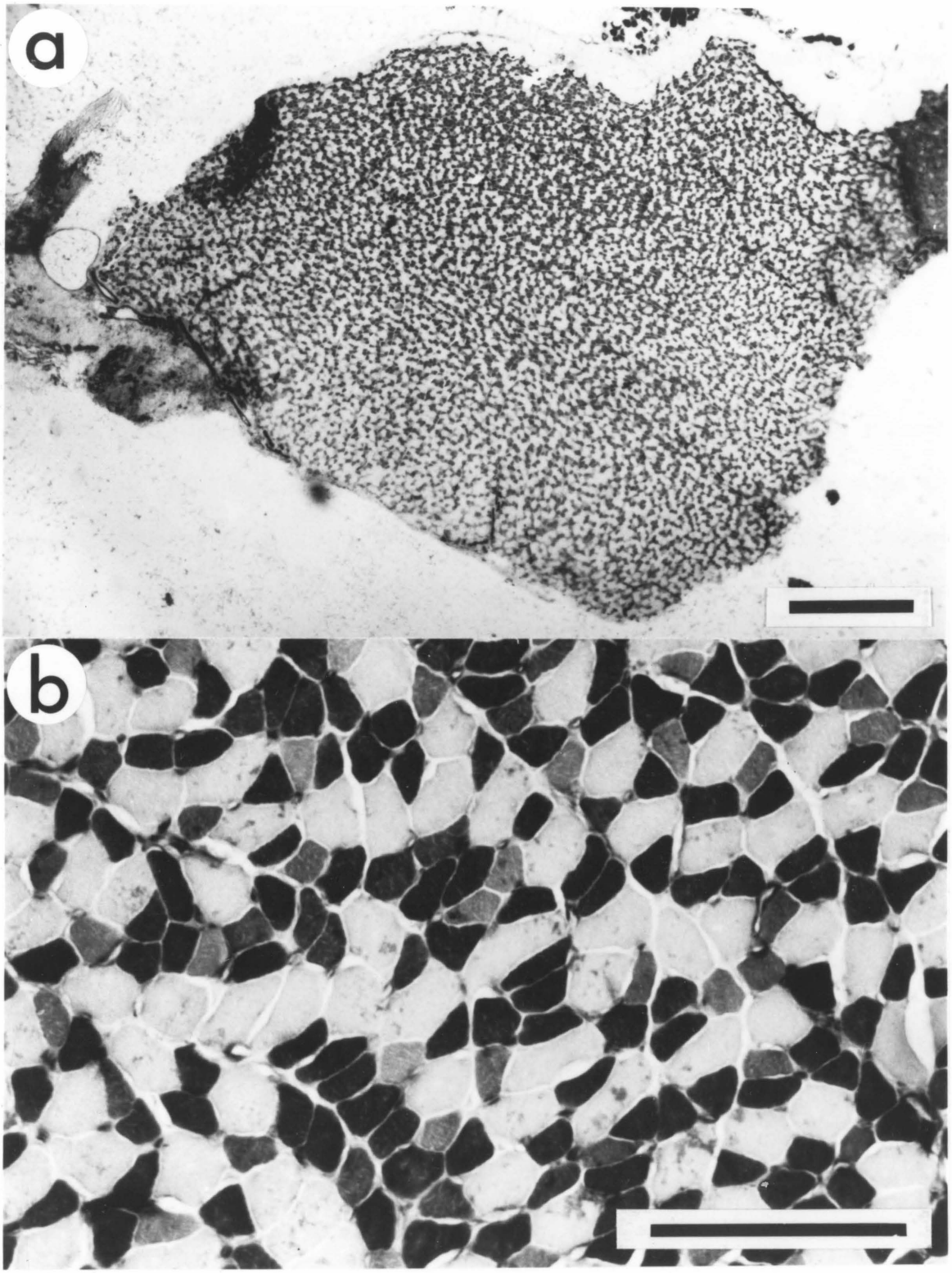
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present at a frequency of only 24% at one margin and 45% at the other margin.

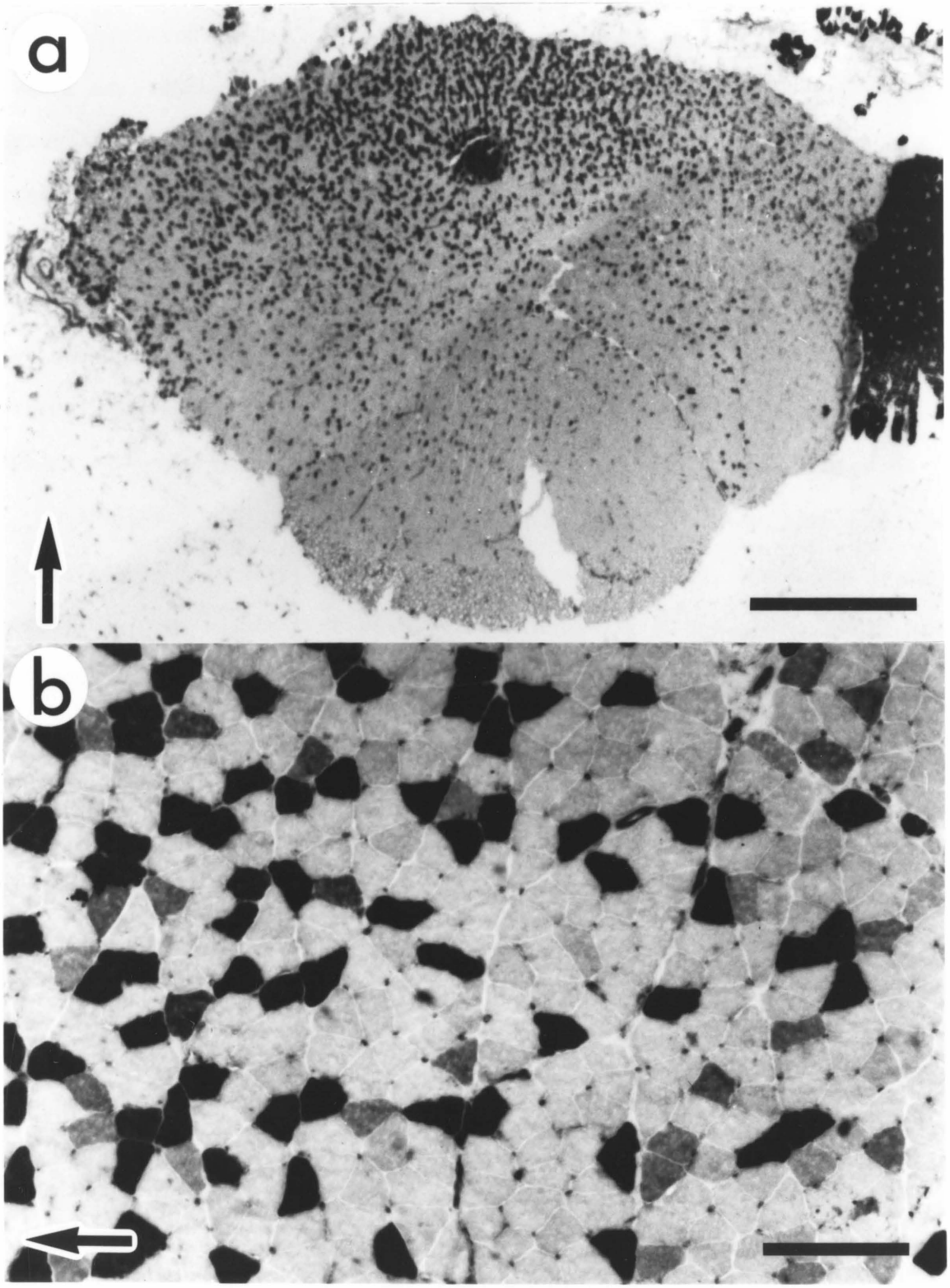
The basic pattern of histochemical staining in muscles from animals of the intermediate age group (Fig. 9) differed in only one fundamental respect from that seen in the early group. In muscles from the older end of this age group, a third intermediate staining fiber type was seen. Otherwise, fiber types were present with the same overall frequencies as were seen in the early group (30% type I, 62% type II, and 7% intermediate), and type I fibers were still about twice as large as type II fibers. For each fiber type, cross-sectional areas varied by between 17% and 33% of the mean. In one muscle examined in detail from a 13-day-old animal (#350), only two fiber types and no spatial gradient across the muscle were found. The cross-sectional areas of muscle fibers as well as of the entire muscle were all relatively small. The 15-day-old muscle (#352) illustrated in Fig. 9 appeared to be developmentally more mature. Cross-sectional areas were two to three times larger than for the 13-day-old muscle, and there was a weak spatial gradient of all fiber types, with type I fibers occurring with a frequency of 20% at the dorsal margin and 32% at the ventral margin. Intermediate fibers followed the same gradient, being 11% at the dorsal margin and 18% at the ventral margin. Type II fibers, however, followed an opposing gradient, being present on the dorsal margin with a frequency of 68% and on the ventral margin with a frequency of only 50%. Intermediate fibers were smallest throughout the muscle ($187 \pm 62\ \mu m^2$ across the entire muscle) while type II fibers were only slightly larger than intermediate fibers ($234 \pm 46\ \mu m^2$), and type I fibers were twice as large as either of the other types ($480 \pm 80\ \mu m^2$).

Considerable development in the fiber frequency and spatial organization of fiber types within the soleus occurred during the three weeks between the intermediate and late age groups. In two 36-day-old muscles examined in detail (one of which is shown in Fig. 10), type I fibers were present with an average frequency of 27%, type II fibers with a frequency of 65%, and intermediate fibers with a frequency of 7%. All three fiber types were within 25% of each other in size in both muscles, and within fiber types the variability in size amounted to no more than 22% of the mean. As illustrated in Fig. 10, there was a marked regionalization in all muscles of the late age group with an average between the two studied muscles of 53% type I fibers on the dorsal margin and over 76% on the ventral margin. Type II fibers, as was the case in younger muscles showing a gradient, occurred in a complementary pattern, being present on the dorsal margin with a frequency of 38% and on the ventral margin with a frequency of less than 16%. A similar pattern was observed in four other muscles examined qualitatively in this age group. The pattern in the one soleus muscle examined from an adult rabbit (ca. 4 kg) was essentially the same, except that type I fibers were even more prevalent, accounting for 78% of the muscle while type II fibers accounted for 20% and intermediate fibers for the remaining 2%.

One denervated and one partially denervated muscle were examined histochemically. The former muscle (#327) was denervated at 1.5 days of age and examined at 13.5 days of age. Fiber frequencies were appropriate to the age (33% type I and 67% type II) as was the size of the type I fibers ($234 \pm 91\ \mu m^2$). There was also a noticeable gradient of type I fibers from 20% at one margin to 43% at the other margin. However, type II fibers had apparently not grown ($39 \pm 21\ \mu m^2$), and type I fibers showed a

Figure 10

Thirty-six-day-old rabbit soleus stained for ATPase following pre-incubation at pH 10.4 (animal #388). (a) Cross section through midbelly of muscle. Dorsal margin appears at top of figure. The large dark spot in the ventral portion of the muscle is an artefact caused by a bubble. On the right of the field is a portion of the gastrocnemius muscle which contains mostly type II fibers. (Scale bar = 1 mm.) (b) Enlargement illustrating the transition between a region high in type II fibers on the left and a region low in type II fibers on the right. Dorsal margin appears at left of figure. Fiber types as indicated in Fig. 9. (Scale bar = 100 μm.)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gradient in size as well as frequency across the muscle (103 ± 46 μm^2 at the less well populated, presumably dorsal, margin and 369 ± 133 μm^2 at the more populated margin). The second muscle (#337A) was partially denervated at 1 day of age and examined at 15 days of age. In this exceptional partial denervation, 42 motor units with an average twitch tension of 5.0% were isolated physiologically. Not only was the muscle fully innervated as judged by the correspondence of nerve evoked and direct muscle elicited twitch tensions, but the two-fold overlap of motor unit tensions indicates extensive polyinnervation. Fiber frequencies and areas were entirely normal, except that no intermediate fibers were present. Thus, both muscle fiber differentiation and the loss of polyinnervation were retarded.

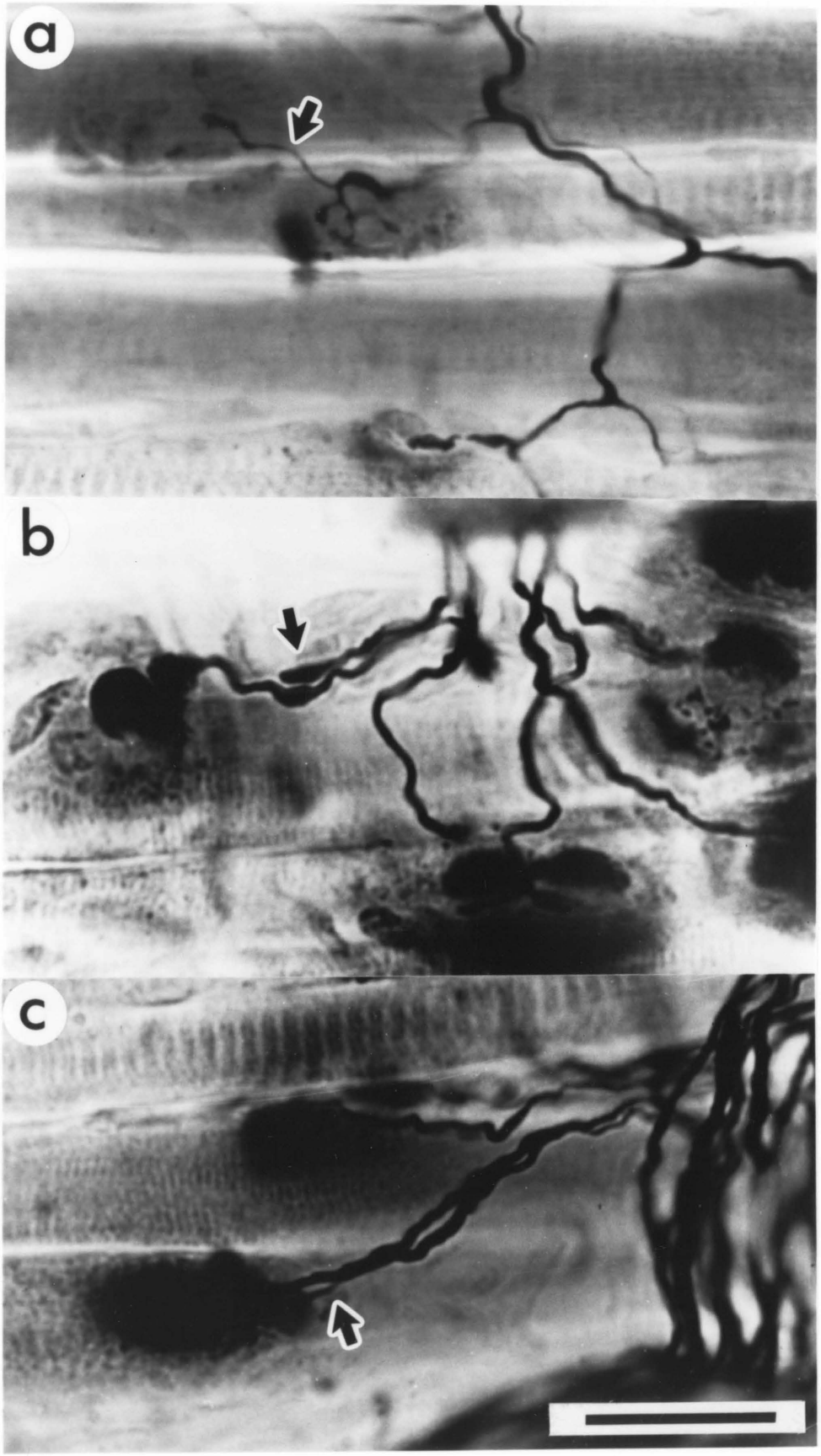
In order to address the anatomical basis of a possible synaptic reorganization following the loss of polyinnervation, motor end plates were examined with a combined silver/cholinesterase stain. Following the loss of polyinnervation several structures indicative of synaptic dynamics were seen with a frequency of 1 or 2 per 100 end plates from about 14 to 21 days of age and with a lower frequency at older ages. The abnormal structures observed included polyinnervated end plates, "retraction bulbs" or growth cones, terminal sprouts to apparently otherwise vacant end plates, and ultraterminal branching (Fig. 11).

Discussion

The number of muscle fibers innervated by a motor neuron at different stages of development can be used as a handle on the changing pattern of neuromuscular connections. To this end, separate estimates of motor unit size for slow and fast twitch motor units were made at the three develop-

Figure 11

Motor axons from a 15-day-old rabbit soleus stained with a silver/cholinesterase stain. (a) A terminal sprout to a vacant end plate and several ultraterminal branches. (b) A retraction bulb or nodal sprout. (c) A doubly innervated end plate. (Scale bar = 20 μm; histology and photography by Michael Brown.)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mental ages of interest in this study. Two different estimates were constructed from different combinations of anatomical and physiological data.

Estimates of Motor Unit Size

The first estimate of motor unit size consisted of dividing the frequency of a given fiber type at a given age by the number of motor units corresponding to that fiber type. The results of this estimate appear in the Table and in Fig. 12. At all ages, type I muscle fibers were considered to correspond to slow twitch motor units and type II fibers to fast twitch units. The number of motor units of each twitch type was calculated using 65 total motor units at all ages (Bixby, Maunsell, & Van Essen, 1980) and the frequency at which slow and fast twitch motor unit types were encountered in physiological experiments (Fig. 2). At the early age both slow and fast motor unit sizes were multiplied by 4.16 to account for the apparent overall degree of polyinnervation. (The scalar, 4.16, was determined from the magnitude of decline in overall average motor unit twitch tension between the early, polyinnervated age group and the intermediate, postpolyinnervated age group.)

The second estimate of motor unit size also used a combination of physiological and anatomical data. In this case, the average motor unit twitch tension for a given motor unit twitch type (Fig. 4) was divided by the relative cross-sectional area and relative specific tension (see Methods chapter) for the corresponding muscle fiber type (Table and Fig. 13). At all ages, the relative specific tension, the tension per unit area, was assumed to be twice as large for fast twitch fibers as for slow twitch muscle fibers. This ratio of specific tensions was chosen because

TABLE: NEUROMUSCULAR DEVELOPMENT OF RABBIT SOLEUS MUSCLE

SUMMARY DATA OF AVERAGE VALUES

Age	1.5-4		11-16			36-43		
Muscle fibers	S	F	S	I	F	S	I	F
Frequency (%)	31	69	30	7	62	65	7	27
Area (relative)	2.2 :	1	1.9 :	0.8 :	1	1 :	0.9 :	1
% of total area	50	50	46	5	50	66	7	27
Motor Neurons	S	F	S		F	S		F
Number	29	36	36		29	33		33
Tension (%) (measured)	5.2	7.6	0.9		2.5	1.9		2.8
Estimated Motor Unit Size								
Fiber frequency (%) / # of motor neurons	4.5	8.0	0.8		2.1	2.0		0.82
Tension/Fiber Area / Specific Tension	3.8	6.0	0.7		1.8	1.88		1.41

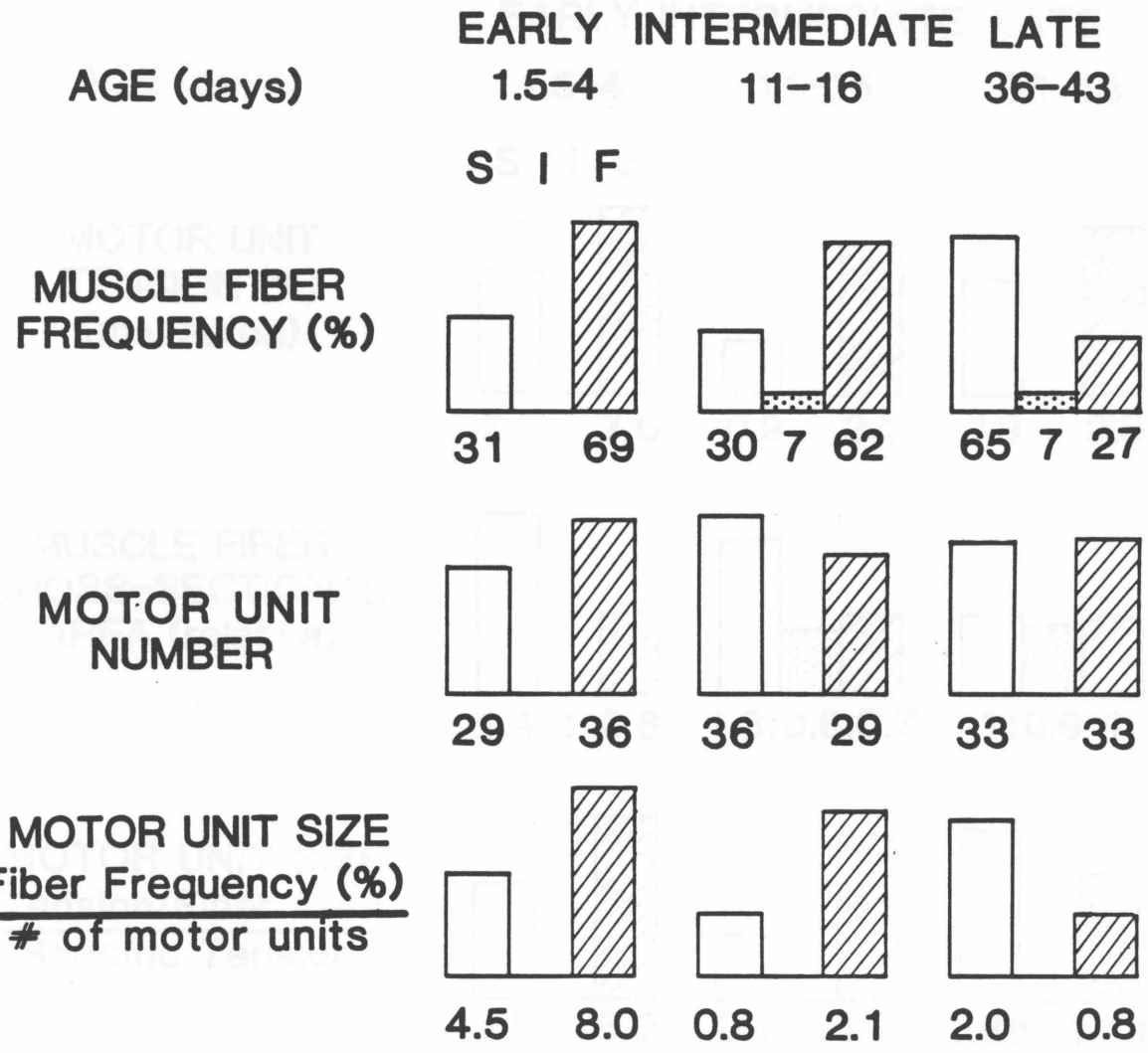
Figure 12

Assessment of slow and fast motor unit size during postnatal development of the rabbit soleus muscle: Method 1. Estimates of motor unit size for each group were made from frequencies of muscle fiber types determined anatomically and from the number of motor units with slow twitch or fast twitch characteristics (Fig. 2) as determined from physiology. Estimated motor unit sizes at the early age were multiplied by 4.16, the apparent degree of polyinnervation present in these animals as determined by comparing median motor unit twitch tensions before and after loss of polyinnervation (Fig. 3). (S = light staining, type I, muscle fiber or slow motor unit; I = intermediate staining muscle fiber; F = dark staining, type II, muscle fiber or fast motor unit.)

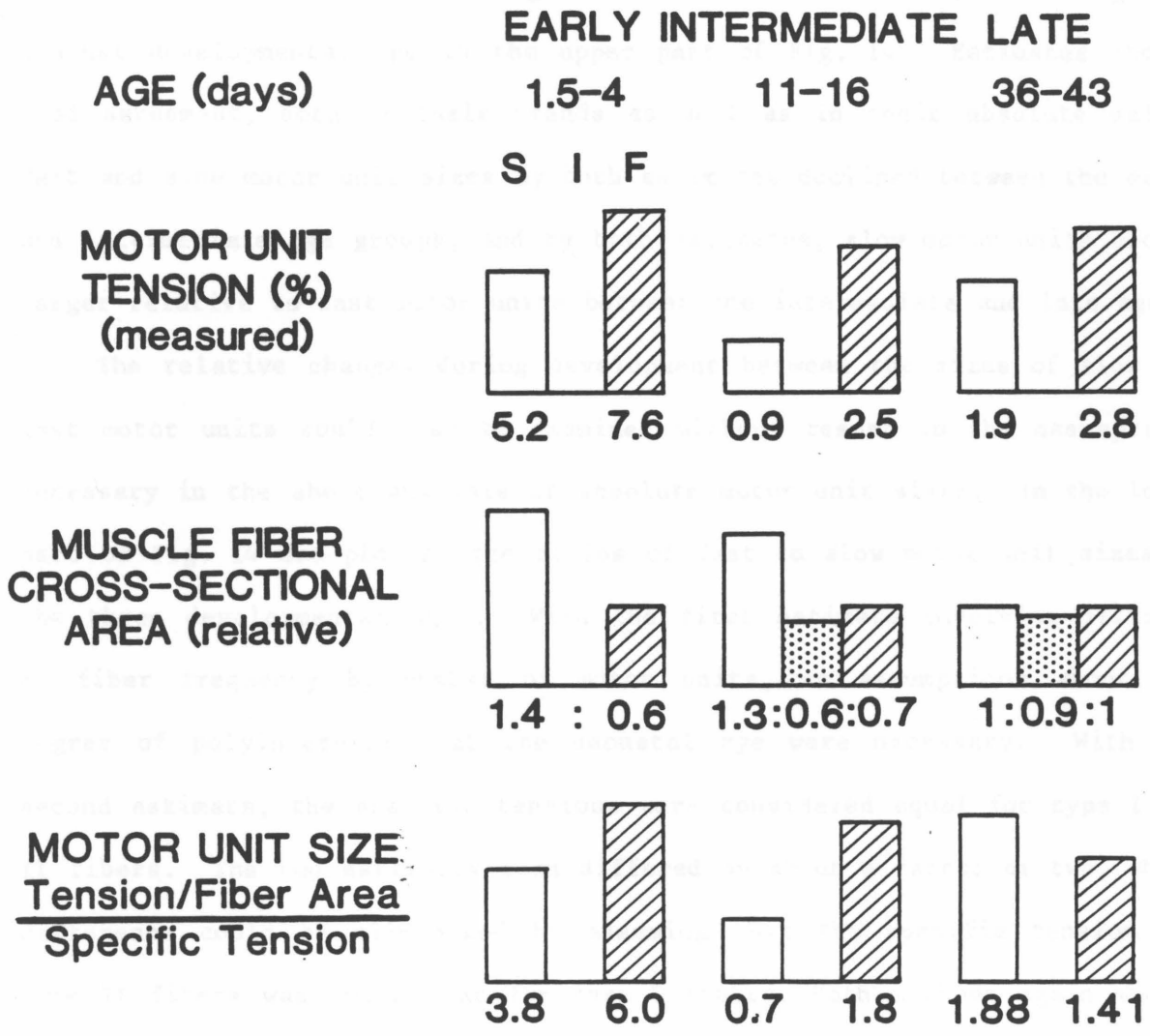
Figure 13

Assessment of slow and fast motor unit size during postnatal development of the rabbit soleus muscle: Method 2. Estimates of motor unit size for each group were made from median twitch tensions of slow and fast motor units as determined physiologically (Fig. 3) and from the relative cross-sectional areas of each muscle fiber type at each age. For all ages, the tension per unit area generated by each muscle fiber, the specific tension, was assumed to be twice as great for fast twitch fibers as for slow twitch fibers. (S, I, & F as in Fig. 13).

ASSESSMENT OF SLOW & FAST MOTOR UNIT SIZE



ASSESSMENT OF SLOW & FAST MOTOR UNIT SIZE



Burke (1981) estimated the specific tension of type I fibers in the cat soleus to be about half that of the type II fibers in the cat flexor digitorum longus. Intrinsic to all the relative areas and specific tensions was a single scaling factor which gave overall average motor unit sizes similar to those determined by the first method.

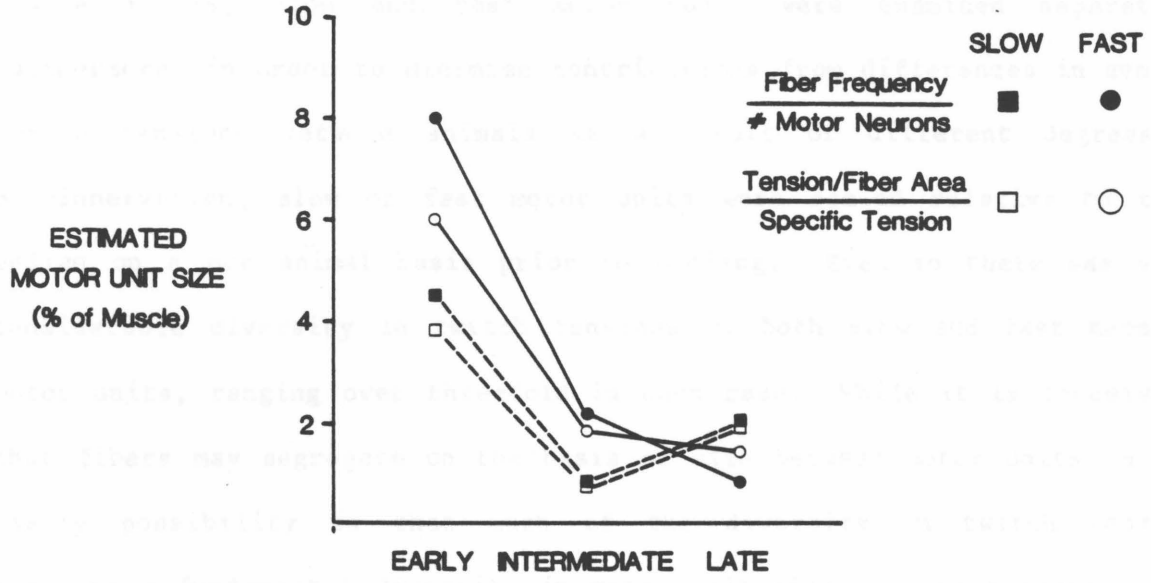
The motor unit sizes arising from both estimates were plotted together against developmental age in the upper part of Fig. 14. Estimates showed good agreement, both in their trends as well as in their absolute value. Fast and slow motor unit sizes by both estimates declined between the early and intermediate age groups, and by both estimates, slow motor units became larger relative to fast motor units between the intermediate and late ages.

The relative changes during development between the sizes of slow and fast motor units could also be examined without resort to the assumptions necessary in the above analysis of absolute motor unit sizes. In the lower part of Fig. 14 are plotted the ratios of fast to slow motor unit sizes at the three developmental ages. With the first estimate involving division of fiber frequency by number of motor units, no assumptions about the degree of polyinnervation at the neonatal age were necessary. With the second estimate, the specific tensions were considered equal for type I and II fibers. The two estimates then differed by about a factor of two, which difference could be eliminated by assuming that the specific tension for type II fibers was twice that for type I fibers. Both methods again showed that the average size of slow motor units declined by more than that for fast motor units during the loss of polyinnervation from the early to the intermediate age. Furthermore, both methods again revealed that in the ensuing weeks, slow motor units became larger relative to fast motor units.

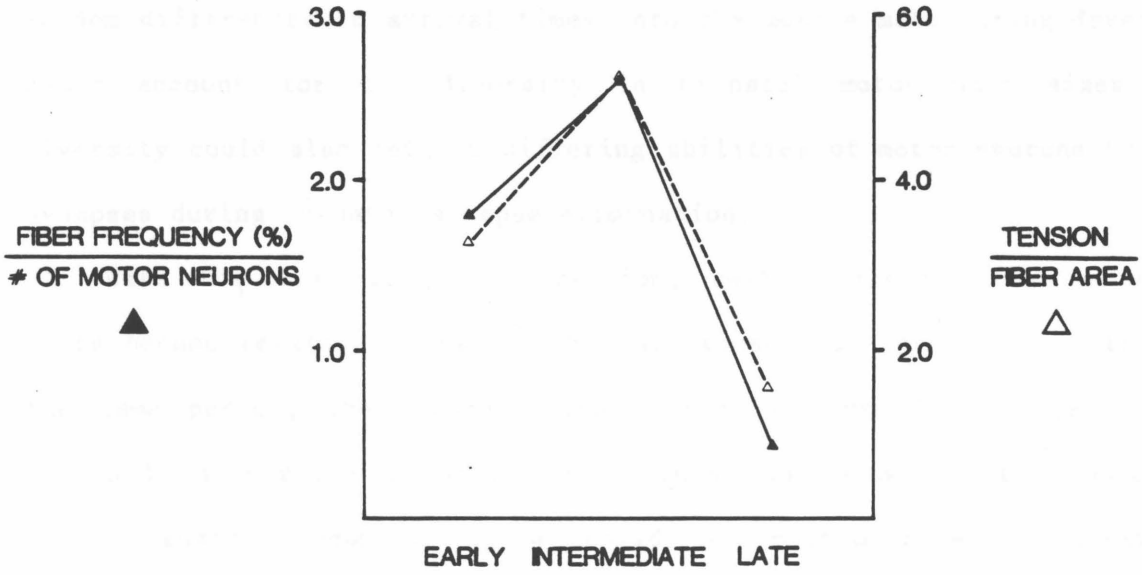
Figure 14

Evidence for exchange of muscle fibers between fast and slow motor units in the rabbit soleus muscle following the neonatal period of polyinnervation. In the top figure are plotted estimated sizes for fast and slow motor units at three developmental ages as determined by two different methods (Figs. 11 & 12). Note that both fast and slow motor units, by both methods, decrease in size from the early to the intermediate age, reflecting the loss of polyinnervation. From the intermediate to the late age, a time when the fibers of this muscle are singly innervated, the size of slow motor units increases at the expense of the size of fast motor units. In the lower figure are plotted the ratios of fast to slow motor unit size at three developmental ages as determined by the two methods of analysis. Note that by both estimates, slow motor units seem to lose more synapses than do fast motor units during the loss of polyinnervation between the early and intermediate ages. Also, from the intermediate to late ages, slow motor units increase in size at the expense of fast motor units.

MOTOR UNIT SIZE DURING POSTNATAL DEVELOPMENT



RATIO OF FAST TO SLOW MOTOR UNIT SIZE



Motor Unit Sizes during the Loss of Polyinnervation

Motor unit twitch sizes showed considerable diversity in the neonate (Fig. 1). In order to minimize contributions to the diversity of twitch sizes resulting from differences in the twitch properties of the component muscle fibers, slow and fast motor units were examined separately. Furthermore, in order to minimize contributions from differences in average twitch tensions between animals as a result of different degrees of polyinnervation, slow or fast motor units were scaled relative to their median on a per animal basis prior to pooling. Even so there was still considerable diversity in twitch tensions of both slow and fast neonatal motor units, ranging over threefold in each case. While it is conceivable that fibers may segregate on the basis of size between motor units, a more likely possibility is that much of the diversity in twitch tensions reflects a fundamental diversity in motor unit sizes in the neonate. Such a diversity may reflect differing abilities of motor neurons to innervate muscle fibers at an earlier stage of development. As simple a reason as random differences in arrival times into the muscle mass during development could account for the diversity in neonatal motor unit sizes. The diversity could also reflect differing abilities of motor neurons to retain synapses during prenatal synapse elimination.

With the loss of polyinnervation, twitch tensions from slow motor units became relatively smaller than did those from fast motor units. Over the same period, the cross-sectional areas of type I and type II fibers remained in a ratio of about 2:1. Thus, the slow and fast motor unit twitch tensions shown in Fig. 4 provide a direct measure of relative slow and fast motor unit sizes at the early and intermediate ages. The diversification in motor unit sizes seen in the rabbit soleus at the intermediate

age in the preliminary study (Fig. 1) is thus largely explained by the increased separation between median slow and fast twitch tensions.

The different degrees of decline in fast and slow motor unit twitch tension during the postnatal loss of polyinnervation can be interpreted in any of three different ways. First, if terminals from slow and fast motor neurons shared a major fraction of the end plates in a polyinnervated, neonatal muscle, then there may have been a competitive elimination in which slow terminals were preferentially eliminated. However, as suggested in the first chapter, terminals from slow and fast motor neurons were largely segregated on their respective fiber types. A second interpretation, then, would be that slow and fast muscle fibers were polyinnervated to different extents in the neonate and that therefore slow and fast motor units necessarily experienced different degrees of synapse loss. As judged strictly by the median twitch tensions of slow and fast motor units (Fig. 3), the average degree of polyinnervation at the early age would have been sixfold for slow motor units and threefold for fast motor units.

A third explanation for the differences in relative decline in slow and fast twitch sizes during the postnatal loss of polyinnervation is that maturation of type II fibers yielded a two-fold increase in specific tension relative to that of type I fibers. The contribution of this last explanation is virtually impossible to assess and highlights the need for a more direct determination of motor unit sizes.

While the diversification in overall motor unit twitch size observed in the preliminary study (Fig. 1) has been largely explained as a relative diversification between slow and fast motor unit twitches, there remains a component of relative diversification among twitch sizes of a given type (Figs. 5 and 6). For both slow and fast motor units, the diversity in

twitch sizes at the intermediate age was larger than it was at the early age. Initially small motor units may thus have been at a competitive disadvantage during the period of wholesale synapse elimination, in contrast to the hypothesis of Brown et al. (1976). Similarly, twitch tensions from the rat soleus as analysed in this study and from the small sample of data presented by Brown et al. (1976) also showed a relative diversification between the early and intermediate ages. It is as if those motor neurons which were less able to originally innervate the muscle were also less able to retain synapses during the first two postnatal weeks. Alternatively, the apparent relative diversification in twitch size may reflect contamination of the slow and fast twitch motor unit pools by motor units of the opposite type.

Secondary Synaptic Reorganization

Several lines of evidence suggest that there is a secondary reorganization of synapses in the rabbit soleus muscle during the three weeks after the loss of polyinnervation. Two different estimates of slow and fast motor unit sizes both showed that over this time period slow motor units became relatively larger in size as compared to fast motor units (Fig. 14). Underlying the shift in slow and fast motor unit sizes was an increase in the strength of slow twitches relative to that of fast twitches (Fig. 3) despite a decrease in the size of type I fibers relative to type II fibers (Fig. 13). Furthermore, there was a conversion of type II fibers into type I fibers without a concomitant change in the frequency of slow and fast motor units (Fig. 12). The simplest explanation for these events is that between the intermediate and late ages, slow twitch motor neurons sprouted new connections onto previously fast twitch muscle fibers

and fast twitch motor neurons relinquished some of their connections to the slow twitch motor neurons.

Even within motor units of a particular twitch type there seemed to be a reorganization of relative sizes. By the late age, the diversity of twitch tensions had declined for both slow and fast rabbit soleus motor units (Figs. 5 and 6). A similar decrease in diversity was seen for rat soleus motor units as well. The hypothesis of Brown et al. (1976), though apparently inappropriate to the period of wholesale synapse loss between the early and intermediate ages, may nonetheless apply to the dynamics of an apparent secondary reorganization of synapses. Those motor units making fewer synapses at the intermediate age seemed to be better able to form new synapses in the secondary reorganization. However, as mentioned above, the small changes in diversity may simply have reflected contamination of the slow and fast twitch motor unit pools by motor units of the opposite type.

The secondary reorganization of synapses was associated with a developing regionalization of muscle fiber types such that by the late age and thereafter, type I fibers were most prevalent on the ventral margin of the rabbit soleus and the remaining type II fibers were most frequent on the dorsal margin. Such regionalization of fiber types has also been reported in cat gastrocnemius (Burke et al., 1977), cat tibialis anterior (Gordon & Phillips, 1953), rabbit superior rectus (Kern, 1966), rat soleus (Kugelberg, 1976), and cat diaphragm (Riley & Berger, 1979). Gordon and Phillips (1953) point out that such a organization within an extensor muscle fits with the scheme of Denny-Brown (1929) that within a group of extensor muscles, there is typically a slowly contracting deep component and a rapidly contracting superficial component. Given the nature of the attachment to a common distal tendon, such an organization grants the

greatest leverage to the more eccentrically placed fast twitch motor units. Thus, there may be a functional advantage to a spatial segregation of motor unit types within the rabbit soleus, but it is interesting that the development of such a pattern is associated with postnatal experience. An analogous development of segregated connections occurs in visual cortex where the initial projections from each eye are initially overlapping, but segregate into ocular dominance columns in the presence of visual experience (Hubel, Wiesel, & LeVay, 1977; Shatz & Stryker, 1978; LeVay, Stryker, & Shatz, 1978; Stryker, 1981; Swindale, 1981).

Muscle fibers undergo a similar conversion from type II into type I in the rat soleus, but do so at a later age. Between 5 and 34 weeks of age the percentage of type I fibers in this muscle increased from 66 to 93 while the incidence of slow twitch motor units also increased from 67% to 90% (Kugelberg, 1977). Kugelberg (1977) argued that slow twitch motor units were transformed during this time period into fast twitch. He found, using glycogen-depletion to identify component fibers, that motor units at 5 weeks of age showed a gradual gradient of contraction times, ATPase reaction intensities, and fiber areas, as if entire motor units were in transition from fast twitch to slow twitch type. Kugelberg's hypothesis of transformation of motor unit types in rat soleus contrasts with that proposed here for rabbit soleus. Whereas he found a higher percentage of slow twitch motor units by 34 weeks of age in the rat, I found essentially no change in the percentage of motor unit types by 5 weeks of age in the rabbit. In the rat, glycogen depleted muscle fibers of individual motor units with intermediate twitch times-to-peak did reveal occasional type IIC fibers, a suspected transitional muscle fiber type (Kugelberg, 1977). Because of the close proximity of the type IIC fibers to other muscle

fibers of a motor unit, Kugelberg conjectured that at least a few muscle fibers might be recruited into a motor unit by "subterminal sprouts". It is possible that such sprouting suggested at 5 weeks of age may have been a remnant of a synaptic reorganization in rat soleus at a time corresponding to the one proposed here in rabbit soleus. After 5 weeks of age, sprouting of axon processes may not be possible, possibly because of growth and formation of connective tissue. Thus, any transformation of the frequency and distribution of muscle fiber types to meet the changing needs of the growing organism could be accomplished only by transformation of motor unit properties rather than by recruitment or loss of component muscle fibers.

Motor neurons are capable of sprouting to and innervating inactive muscle fibers throughout life (Brown et al., 1981). What is not well established is whether sprouting can occur to innervated, active muscle fibers or whether existing inputs to muscle fibers ever withdraw or become dysfunctional, thereby inviting cross innervation. Invasive experiements in which a foreign nerve was placed over the end plates of an innervated rat soleus muscle demonstrated that the original synapses could be displaced by foreign sprouts (Bixby & Van Essen, 1979b). However, do motor neurons sprout and exchange muscle fibers during normal life? In the frog, a secondary reorganization of synapses within and between muscle fibers seems to occur in adult pectoral muscle (Haiman et al., 1981). There is also some anatomical evidence of synaptic rearrangements in adult mammals (Kemplay & Stolkin, 1980). Most notably, Barker and Ip (1966; their Fig. 4) show a terminal branch to a neighboring fiber in adult rabbit soleus muscle. On the other hand, Tuffery (1971) failed to find collateral sprouting in cat soleus.

Two techniques were applied to the rabbit soleus from animals between 11 and 36 days of age in hopes of documenting end plates in a polyinnervated transition state. Intracellular physiology of end plate potentials revealed no evidence of polyinnervation. However, for technical reasons, a low incidence of polyinnervated end plates could easily have been missed. Silver cholinesterase stains revealed a low incidence of peculiar periterminal axonal structures (Fig. 11), particularly between 2 and 3 weeks of age. If exchange of end plates between motor neurons occurred in as little as one day, then one might not expect to find evidence for polyinnervated terminals with any but a very small frequency. Hence, the few observed anatomical structures are at least consistent with the hypothesis of synaptic reorganization.

In conclusion, several lines of evidence suggest that a reorganization of synapses occurs in the soleus muscle of the rabbit after the loss of polyinnervation. In the course of this reorganization, end plates on type II muscle fibers in the ventral portion of the muscle are preferentially exchanged for innervation from slow twitch motor neurons. During the course of the synaptic reorganization, there may be a slight preference for the retention and formation of synapses by motor neurons with fewer existing synapses.

Appendix

TEST FOR A COMPETITIVE ROLE OF ACTIVITY IN SYNAPSE ELIMINATION

Activity has been found to play a role in the development of mammalian neuromuscular connections. The developmental loss of polyinnervation is delayed by blockade of neuronal activity (Benoit & Changeux, 1975, 1978; Thomson, Kuffler, & Jansen, 1979). In a complementary fashion, increased activity speeds the loss of polyinnervation (O'Brien, Östberg, & Vrbova, 1978; Thompson, 1983). Furthermore, activity appears to regulate the existence of polyinnervation in an *in vitro* neuromuscular system (Fishman & Nelson, 1981). While the role of activity in regulating the presence or absence of polyinnervation and the rate at which polyinnervation is lost during normal development is well documented, its competitive role is still open to question. At an individual polyinnervated end plate, is an inactive terminal more likely to be eliminated in development that an active one? To address this issue, I and David Van Essen blocked the activity in a small subset of motor axons contributing to the polyinnervated neonatal rabbit soleus muscle. Because of the polyinnervation and the presence of many active motor axons, virtually all of the muscle fibers remained active. Thus, active motor terminals competed with inactive motor terminals on active target muscle fibers.

Methods

The impulse activity of spinal root S2 contributing to the soleus muscles of rabbits 4 to 5 days of age was blocked for 4 to 5 days by surgical insertion through the perineurium of a slowly releasing plug containing tetrodotoxin (TTX). (See Methods chapter for details.) Four animals were treated with TTX-containing plugs and seven with plugs containing only vehicle solution. The period in development during which

plugs were administered corresponded to the maximal rate of loss of polyinnervation in the rabbit soleus (Bixby & Van Essen, 1979a). The specific blockade of the motor axons within spinal root S2 was produced by this paradigm without apparent damage as judged by visual inspection of the nerve at the plug site and by the consistent conduction of electrically induced impulses through the plug site as observed following removal of the plug, at the time of the physiological assay. A limited amount of axotomy resulting from the trauma of plug insertion into the nerve would have had no great consequence on the interpretation of the results. At the time of insertion, the muscle was heavily polyinnervated, and a limited loss of axons would still have left most of the muscle fibers polyinnervated. The short duration of the impulse blockade, furthermore, left no possibility of regrowth of severed axons into the muscle.

The competitive success of terminals of active and effectively inactive motor neurons was measured with a physiological assay of motor unit twitch strength. (See Methods chapter.)

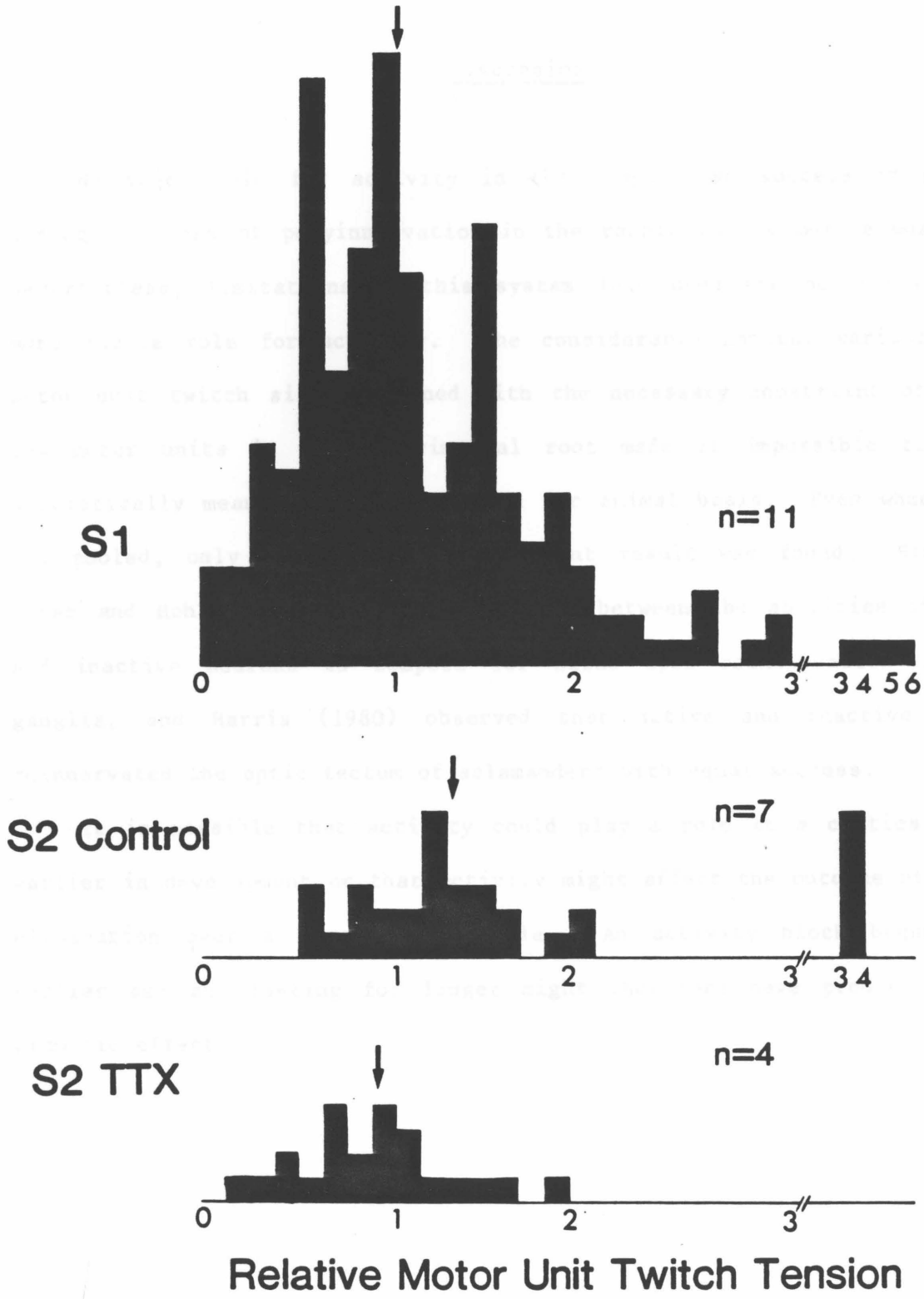
Results

Twitch tensions of motor units from spinal roots S1 and S2 were scaled relative to the median of spinal root S1 for each animal and then pooled separately by root. The results for spinal root S2 were separately pooled according to whether it had been treated with a plug containing TTX or vehicle solution (Fig.). On a per animal basis there was no significant difference by parametric statistics in the mean motor unit twitch size nor by nonparametric statistics in the median between control and experimental roots. When the results were scaled and pooled, the average scaled motor

Figure

Pooled motor unit twitch tensions from (top) untreated reference spinal root S1, (middle) control vehicle solution plugged spinal root S2, and (bottom) TTX plugged spinal root S2. Twitch tensions from motor units of spinal root S1 were scaled on a per animal basis to their median and pooled. Twitch tensions from motor units of spinal root S2 were then expressed on a per animal basis as a fraction of the median motor unit twitch tension for spinal root S1. Arrows indicate the median of each distribution.

Role of Activity in Synapse Elimination



unit twitch size, however, was significantly different by parametric statistics (0.92 ± 0.08 [s.e.m.] for TTX-treated motor units vs. 1.2 ± 0.07 [s.e.m.] for control-treated motor units).

Discussion

No major role for activity in the competitive success of synapses during the loss of polyinnervation in the rabbit soleus muscle was found. Nevertheless, limitations in this system left open the possibility of a more subtle role for activity. The considerable natural variability in motor unit twitch sizes combined with the necessary constraint of only a few motor units in the experimental root made it impossible to obtain statistically meaningful results on a per animal basis. Even when scaled and pooled, only a marginally significant result was found. Similarly, Roper and Hoh (1978) found no difference between the abilities of active and inactive neurons to compete for sites upon reinnervation of heart ganglia, and Harris (1980) observed that active and inactive neurons reinnervated the optic tectum of salamanders with equal success.

It is possible that activity could play a role at a critical period earlier in development or that activity might affect the outcome of synapse elimination over a longer time scale. An activity block begun at an earlier age and lasting for longer might therefore have produced a more dramatic effect.

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