REGULATION OF COMPETITIVE INTERACTIONS DURING NEUROMUSCULAR SYNAPSE ELIMINATION

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Table of Contents

Acknowledgements iii

Chapter 1. General Introduction 1

Chapter 2. Slowing of Synapse Elimination by
α-Bungarotoxin Superfusion of the Neonatal
Rabbit Soleus Muscle 19

Chapter 3. Differential Loss of Neuromuscular
Connections According to Activity Level and Spinal
Position of Neonatal Rabbit Soleus Motor Neurons 56

References 141

CHAPTER 1

General Introduction

Given the simple knowledge that development begins with a single undifferentiated cell and can result in an organism as complex and multipotent as a human, there is no escaping the realization that an incredibly complex, precise, and reproducible process must occur. Probably the most complex system to develop in this process is the nervous system. The century-old studies of Ramon y Cajal (1890) attest to the remarkable diversity of neuron types, to the great potential of neurons in driving complex behavior, and to the awesome problem that one faces in trying to understand how such a complex machine is put together.

A full understanding of nervous system development must include, as a minimum, a comprehension of how each of the broad classes of processes enumerated below is regulated. The first problem for the developing organism is that a small number of undifferentiated cells must proliferate and become specified as neurons *per se*, and these neurons must be parsed into the multitude of subclasses of neuronal types. Once a neuron is born, it must attain its proper position in relation to other cells and finally send processes to, and select from, amongst a myriad of potential neuronal or non-neuronal targets. Neurons must also respond to the signals they receive from others in a manner that is appropriate for their identity and function. At each of these stages, decisions must be, and are, made with great precision.

Despite the capacity for precision in neuronal development, mammalian systems do not adhere in all cases to an inexorable plan. This is in sharp contrast to less complex organisms, especially invertebrates, with the nematode *C. elegans* serving as the classic example of rigidly controlled development. (Yet even the nematode has the capacity to alter its developmental program in response to perturbation, Chalfie et al., 1983.) Development in higher organisms appears to mimic phylogeny – the most inflexible processes occur early and the apparently more evolved and regulative processes occur later. The later occurence of more

malleable processes may reflect an evolutionary trend necessitated by the greater complexity and attendant increased potential for error in higher organisms; in a regulated system, errors can be corrected. Another possibility is that some decisions are best postponed until postnatally, when interaction with the environment is able to impose meaningful activity patterns on neural elements that may then respond in an adaptive manner.

There are two principal stages when connectivity is reworked in the development of the mammalian nervous system. The first occurs before birth, and around the time of synapse formation, involving the death of a large fraction of the initial neuronal population. It is apparently regulated such that the neuronal population is trimmed to a size appropriate to the size of the target (see Cowan et al., 1984 for review). The second is also a degenerative process, involving the elimination of many of the connections that were made during the initial period of synapse formation, but without the death of the parent cell. In some systems, neurons lose connections and make new ones concurrently; these processes are thus best considered a rearrangement of connectivity. It is the second of these "regressive" stages that is the focus of this thesis – in particular, the elimination of neuromuscular synapses that occurs in neonatal mammals. This chapter will introduce some of the major issues in regulation of neuromuscular synapse elimination and will consider how the type of regulation and apparent goals of the process might make a phase of postnatal rearrangement a suitable means of achieving those goals. In these contexts, similarities and differences with other neuronal systems that undergo postnatal rearrangement of connections will be considered.

Development of precise single innervation

At birth, mammalian muscle fibers are innervated at a single endplate by several different motor neurons. In a matter of a week or two, nearly all of the redundant

connections are eliminated, apparently by retraction of presynaptic terminals into parent axons (Bixby, 1981; Riley, 1981), and the adult state in which each endplate is innervated by exactly one motor neuron is achieved (Brown et al., 1976). Similar phenomena, in which multiple innervation is reduced to precise single innervation, also occur in other parts of the nervous system. The best example of such a system is climbing fiber input to cerebellar Purkinje cells – precise single innervation is achieved at every Purkinje cell (Mariani and Changeux, 1981). For other systems, such as the rat submandibular ganglion (Lichtman, 1977), the majority of the postsynaptic cells become singly innervated, but a small fraction retain input from two or more neurons. During the formation of ocular dominance columns in layer 4 of striate visual cortex (Hubel and Wiesel, 1963), most of the postsynaptic cells become exclusively innervated by inputs corresponding to a single eye. This can be considered single innervation in a functional sense, if the cells responding to visual stimulation from the same eye and at similar retinotopic positions are considered a single presynaptic element.

Evidently, the occurrence of more than one synapse at a single mammalian neuromuscular endplate is not stable. Experiments in which a foreign nerve is transplanted onto a muscle suggest that this instability may be related to the spatial proximity of the synapses (Brown et al., 1976; Kuffler et al., 1977). Following denervation, mammalian muscle fibers become susceptible to innervation by transplanted foreign innervation. The foreign nerve often forms a synapse and induces new postsynaptic specializations at a site other than the original endplate. If the original nerve or a second foreign nerve is also allowed to reinnervate the muscle, endplates can occur at more than one position on a single muscle fiber, thus allowing a test of whether interactions between separated connections, possibly mediated by the common postsynaptic cell, can occur. The stability of multiple endplates on

a single muscle fiber is appparently closely related to the distance between the endplates, since one of the redundant endplates is removed when they are within a few millimeters of one another, but the multiple endplates are relatively stable if they are separated by longer distances.

While this result suggests the possibility of muscle fiber-mediated interaction between distant presynaptic elements, the type of interaction could be quite different from any that normally occur during the elimination of focal multiple innervation. For example, suppose that the loss of a redundant endplate represents instability of dual postsynaptic elements rather than their multiple presynaptic inputs. That such instability may exist is evidenced by the observation that during synapse formation, twitch muscle fibers become refractory to induction of postsynaptic specialization at a second site, following the formation of an original endplate. The occurence of two endplates after transplantation of foreign nerves is not normal, but once it does occur, the muscle fiber may take measures to eliminate the redundant set of postsynaptic specializations or simply may be unable to maintain both. The loss of the presynaptic terminal would be likely follow.

Kuffler et al. (1977) suggested that the instability was in fact related to the multiple presynaptic elements, based on the observation that esterase-stained endplates void of terminals occur in muscles after reinnervation by foreign nerves and subsequent elimination of synapses. Thus, they proposed that the elimination of synapses in their preparation was similar to the normal process, involving retraction from stable postsynaptic specializations. However, this is not necessarily the case. It is possible that postsynaptic elements that maintain the stability of terminals do not persist as long as esterase or that the empty esterase sites represent original endplates that were never reinnervated. In an *in vitro* model described by Magchielse and Meeter (1986), a similar removal of redundant endplates is induced

by stimulation. The ability of this system to be considered an adequate model of synapse elimination in mammalian skeletal muscle is limited by the inability to induce the state of focal multiple innervation that is the rule *in vivo*.

The observation that a redundant endplate can be stable, if a separation of more than a few millimeters occurs, suggests that the distance over which interaction can occur is limited. Interactions also appear to be spatially restricted in other parts of the peripheral nervous system; the adult number of synapses per postsynaptic cell following postnatal rearrangement in the rabbit ciliary ganglion (mean of about 6) is closely related to the complexity of dendritic arborization (Purves and Hume, 1981). This observation suggests that similar mechanisms might be at work in systems whose final goal is single innervation and those that maintain polyinnervation into adulthood.

Evidence for competition

Perhaps the best evidence that neuromuscular synapse elimination is a competitive process comes from partial denervation experiments. If the potential for competition between motor neurons is reduced by severing the axon of most of the motor neurons, the remaining neurons retain a much greater fraction of their original connections than is normal (Brown et al., 1976; Fladby and Jansen, 1987). Competition is also the most straightforward explanation for the observation that single innervation is achieved without a transient period of denervation (Brown et al., 1976). Since single innervation is achieved for most of the postsynaptic cells in some of the systems considered above, it is quite likely that they are also regulated by a competitive process. This is certainly the case for the formation of ocular dominance columns, in which there is more direct evidence for competition (see below).

Although partial denervation experiments strongly suggest that competition between motor neurons occurs, it is not clear from those experiments whether the competition is restricted to local interactions at individual endplates (or at least restricted to individual muscle fibers). Alternatively, indirect interactions between fibers might influence the competition. The simple observation that single innervation occurs without the appearance of denervated endplates strongly suggests, however, that the competitive interactions are at least partially localized. There is some evidence that non-local competition, possibly mediated by diffusible substances, occurs after single innervation is achieved; but this process, known as secondary reorganization, is likely regulated by a different set of rules than synapse elimination (Gordon et al., in preparation). Non-local (indirect) interactions during synapse elimination might be mediated by diffusible substances or by the axonal arbors that span large regions of the muscle. For example, if a terminal from a particular motor neuron is competing poorly at one endplate, it might divert supplies from that terminal to enhance the probability of survival of some other terminal. Diversion of materials away from retracting axonal branches has in fact been demonstrated (Goldberg and Schacher, 1987). Also, trophic substances obtained by one of a neuron's terminals could increase the probability of survival of its others.

One possible adaptive purpose for regulation by a competitive mechanism is that it can assure one-to-one connectivity (whether that be at the level of the individual neuromuscular endplate or a restricted portion of a postsynaptic cell). However, one can conceive of several additional scenarios in which a competitive process would allow better control than could occur by another mechanism. For example, some motor neurons might be better matched to particular postsynaptic cells than others, or it might be preferable for some motor neurons to retain a larger percentage of

their connections than others. Each of these would require a non-random process in which, at the minimum, qualitative decisions are made about which of the multiple inputs to a given endplate should have the greater probability of survival. There is considerable evidence, some described below and some in subsequent chapters, that this sort of regulation does occur.

Relations to topography and error correction

There is good evidence for matching between specific neuronal and muscle populations. Motor neurons are present in distinct motor pools consisting of neurons that innervate fibers from only a single muscle, and some muscles are topographically organized such that there is a systematic correspondence between muscle fiber position and the spinal position of the motor neurons connecting to the fibers (Laskowski and Sanes, 1987a). In the first example of directed elimination of neuromuscular synapses, Brown and Booth (1983) demonstrated that topographic relationships are refined during synapse elimination in the rat gluteal muscle. There is now evidence that a similar refinement also occurs in the lateral gastrocnemius muscle of the rat (Bennett and Ho, 1988).

A common denominator in all of the known topographically organized muscles is that the muscle fiber geometry could potentially reflect different functional roles for different subsets of muscle fibers. These muscles are generally sheetlike, and thus the force generated by the fibers is not isolated to a single site by a shared tendon, but is distributed across a relatively large area. In this sense, fibers at different positions in such muscles can serve different functions, and the improvement of topographic relations during synapse elimination at such muscles might be considered an error-correcting mechanism. Perhaps the mechanisms for generating specificity during synapse formation are adequate for making gross distinctions between different muscles, but the finer distinctions that must be made to generate topography within

a muscle cannot be made at that stage.

Chapter 3 describes a position-dependent differential loss of synapses for the rabbit soleus. The fibers in this muscle share a single proximal and a single distal tendon, each of which has a restricted site of insertion onto bone, and the muscle is likely not to be topographic. The similarities of this phenomenon to differential loss in topographic muscles and the extent to which the phenomenon can be considered an error-correcting mechanism are considered in the discussion of Chapter 3.

Refinement of topography or error correction during postnatal rearrangement occurs in many other parts of the nervous system. Aberrant connections in the central nervous system are removed in the cases of callosal projections and projections from occipital cortex to the pyramidal tract (see Cowan et al., 1984, for review). Examples of topographic refinement include redistribution of axonal arbors from retina to tectum, following grossly retinotopic regeneration of the optic nerve in adult frog (Gaze and Keating, 1970) or goldfish (Meyer, 1980). While refinement of topography is similar to removal of aberrant connections in the sense that both are manifested by elimination of projections to less appropriate postsynaptic partners, for topographic refinement, the level of the rearrangement is much finer, involving selection between neighboring and functionally similar targets rather than distant, apparently unrelated targets. Nevertheless, similar mechanisms may be responsible for insuring the elimination of grossly inappropriate connections and for refinement of topographic relations (i.e., positional or target-specific, molecular markers, or activity-mediated competitive interactions).

Activity dependence

That activity might be important in the regulation of neuronal connectivity was first proposed by Hebb (1949) nearly 40 years ago. Based on the simple observations

that repetition reinforces learning and that memories tend to be associative, he postulated that learning might be mediated by selective strengthening of connections when pre- and postsynaptic activity are correlated. Thus, pathways that were used once would tend to be used with increasing frequency, given identical stimuli, and multiple connections to a common postsynaptic cell would tend to be most stable if they were activated simultaneously.

Although the hypothesis must have been considered largely speculative at the time, it was and still is an extremely fascinating one. If the pattern, or even the strength of connections between neurons could be altered based on activity cues from the environment, it would imply that the nervous system is not an exact hard-wired copy built according to some genetic blueprint. On the ageless question of nature versus nurture, one could not argue that nurture was unimportant, and for those who already believed there was some balance between the two, that balance would probably be shifted a good deal more toward the side of nurture.

Since Hebb formulated his hypothesis, activity has, in fact, been shown to influence neuronal connectivity both in developing systems and in adult systems apparently associated with learning. The first such demonstration was in the developing visual system. Hubel and Wiesel (1963) showed that the number of binocularly driven cells in layer 4 of striate cortex declines dramatically after birth, apparently because of segregation of afferents from different eyes onto separate columns of neurons. If one of the eyes was occluded, the normal segregation of inputs was altered such that the deprived eye maintained fewer connections than normal (Hubel and Wiesel, 1963). In strabismic cats, more pronounced segregation of inputs developed; the columns of monocularly driven cells extended beyond the boundaries of layer 4, apparently owing to the decreased correlation of activity between the two eyes (Hubel and Wiesel, 1965). These results suggested that ocular

dominance column formation is not only activity-dependent, but may be Hebbian. These early observations also served to fuel the fascination with activity begun by Hebb and assured the continued study of the segregation of visual inputs, as well as tests for activity effects in many of the systems that undergo rearrangement and were yet to be discovered.

During the last two decades, continued study of ocular dominance column formation has made a very strong case for Hebbian regulation in that system (see Singer, 1987 for review). To select just a few of the more telling observations: The normal segregation of inputs was prevented if all impulse activity was blocked by intraocular injection of tetrodotoxin (Stryker and Harris, 1986), and segregation occurred despite the blockade if the optic nerves were electrically stimulated; however, if the stimulation was always correlated for the two optic nerves, segregation did not occur (M.P. Stryker, personal communication); in one of the most recent studies, Reiter and Stryker (1987) reported that in monocularly deprived animals, the deprived inputs outcompted the non-deprived if activity was prevented for the postsynaptic cortical cells. This result apparently implies that presynaptic activity in the absence of postsynaptic activity can have a negative influence; of course, the possibility of positive influences resulting from correlated activity, as suggested by the lack of segregation in the correlated stimulation paradigm above, is not ruled out.

In a system more closely matched to Hebb's motivation in formulating his hypothesis, the role of activity has been studied in an *in vitro* model for learning, long-term potentiation (LTP) in the hippocampal slice preparation. Kelso et al. (1986) reported that LTP, like ocular dominance column formation, was not dependent simply on the amount of activity at a synapse, but on the temporal correlation between the pre- and postsynaptic activity. LTP occurred only for

synapses with correlated pre- and postsynaptic activity. If the normal generation of an action potential at the postsynaptic cell was replaced by a simple depolarization and no action potential, LTP still occurred. Since a literal interpretation of Hebb's hypothesis is that simultaneous "firing" of pre- and postsynaptic cells should result in synaptic strengthening, the hippocampal system may not be strictly Hebbian. However, in light of present knowledge of the events that accompany activation of the postsynaptic cell, a broader interpretation of Hebb's postulate seems more sensible. Nevertheless, the distinction between whether postsynaptic spikes are necessary to induce synaptic strengthening or not may prove to be an important one in future classification of systems that are Hebb-like.

It has been known for over a decade that activity levels can profoundly influence the rate of neuromuscular synapse elimination (see Thompson, 1985, for review). In general, increased activity results in faster elimination whereas inactivity has the opposite effect. However, despite the advantage of greater accessibility to the neuromuscular synapse, there are not particularly accessible points where activity can be selectively altered for competing presynaptic elements, e.g., separate retinae in the visual system. Thus, most of the experiments in the neuromuscular preparation have involved altered activity levels for the entire population of pre- or postsynaptic cells (and often both, but see the discussion of Chapter 3 for exceptions).

For this reason, it is important to distinguish between two broad classes of experiments used to test for activity effects at the neuromuscular junction, as well as in other systems: 1) those in which activity levels are altered equally for the entire population of pre- or postsynaptic cells (or both), and 2) those in which activity is altered differentially (again pre- or postsynaptically or both). It is important that one be aware of the limitations inherent to interpretation of observations based on

these types of experiments. For the first type, a positive result (an alteration in the rate of synaptic rearrangement) need not imply that the competitive capability of synapses is activity-dependent or even that competitive interactions occur, only that activity can influence the rate of rearrangement.

A differential change in activity on the other hand, has the potential to test for differences in the competitive ability of more versus less active synapses. It should be noted, though, that a differential outcome need not necessarily imply differential competence; if the elements of the system at which the difference was manifested were not in competition, the result could still reflect a simple influence on the rate of rearrangement. For example, in the experiment described in Chapter 2, there was a differential block of postsynaptic activity, and this resulted in a different rate of synapse elimination, apparently related to the amount of postsynaptic activity blockade. While an advantage to neurons innervating the less active endplates cannot be ruled out, a more conservative interpretation is that this result reflects a difference in the rate of synapse elimination according to the amount of postsynaptic activity. To make strong conclusions, one must differentiate between effects on the rate of synapse elimination versus the competence of particular synapses.

These issues arise again with respect to results from a differential presynaptic activity block described in Chapter 3. A differential block of presynaptic activity must also differentially alter postsynaptic activity levels, since it is the presynaptic that triggers postsynaptic activity. Thus, it was important to rule out the possibility that the difference in maintenance of presynaptic terminals did not result from a simple alteration in the rate of synapse loss at affected endplates. These issues are discussed in greater detail in the context of the specific results in the subsequent chapters, but they are addressed as a specific example here to point out that care must be taken in interpreting these sorts of results.

In this regard, it is of interest to consider once again the refinement of retinotopography in regenerating goldfish tectum as a specific example. In the goldfish retinotectal system, refinement of topography does not occur if impulse activity is blocked by tetrodotoxin (Meyer, 1983). But as Meyer points out, this could result from a failure of axonal arbors to become restricted in size or from a normal amount of arbor restriction but at inappropriate positions; it cannot be determined from this result alone whether the inactivity prevents reorganization or prevents its specificity. Therefore, although it is tempting to assign a role to correlated activity in determining synaptic competence based on the observation that neighboring retinal ganglion cells have correlated activity in the dark (Arnett, 1978), it is also plausible that chemical markers thought to be present at goldfish tectum (Sperry, 1963) confer a competitive advantage to the better matched synapses.

Overview

While many systems in which postnatal rearrangement occurs have not been extensively studied, I know of no such system in which the process has been shown not to be competitive. In addition, in each adequately studied system, the competitive process corrects errors, is activity dependent, or both (i.e., refinement of topography is activity-dependent and can be considered to be error-correcting). Thus, for each of the systems, the apparent advantage of rearrangement is likely to be related to the considerations that have already been proposed above. Namely, as nervous system complexity grows, the potential for developmental error increases, as does the amount of genetic information required for specificity; these factors may necessitate rearrangement as a compensatory mechanism. Additionally, some developmental decisions might depend on environment- and/or use-dependent activity cues, necessitating their delay until postnatally.

These common features do not, however, imply that rearrangement is controlled identically in every system. On the contrary, the great diversity in both function and evolutionary age of systems that undergo rearrangement makes identical regulation unlikely. A particular example of such a difference is described in Chapter 3. Briefly, the sign of activity influence on synaptic competence appears to be opposite for ocular dominance column formation and neuromuscular synapse elimination. This may very well reflect the different goals of the systems: the segregation of inputs with uncorrelated activity patterns in layer 4 of visual cortex (Hubel and Wiesel, 1965), and larger motor units for higher threshold motor neurons in the neuromuscular system (Henneman and Olson, 1965). Mechanistic considerations and adaptive strategies are each considered in further detail in discussion sections of the subsequent chapters of this thesis.

Summary of thesis chapters

The subsequent chapters of this thesis address a number of issues related to the phenomena that occur during neuromuscular synapse elimination and to the rules and mechanisms that govern them. The results they describe are therefore based on observations of developmental processes in both normal animals and in those whose normal developmental interactions have been perturbed by alteration of activity for some of the elements involved.

Chapter 2 addresses the question of whether the rate of neuromuscular synapse elimination might normally depend on the level of postsynaptic activity. Previous studies had strongly implicated activity in regulation of synapse elimination rate; e.g., increased activity evoked by chronic stimulation increased the elimination rate; but these studies did not differentiate between pre- and postsynaptic activity. Duxson (1982) treated the rat soleus muscle with α-bungarotoxin (α-BGT), completely blocking postsynaptic activity during the normal period of synapse

elimination and reported that the number of terminal profiles per endplate observed in electron micrographs did not decrease as it does normally. I did not consider this study to be conclusive because complete activity blockade might invoke influences that are not normally present in active muscles during synapse elimination, and because the assay was indirect – an increase in the number of terminal profiles per endplate might reflect an increase in terminal complexity rather than maintenance of more terminals.

For the experiments described in Chapter 2, postsynaptic activity was partially blocked by α-BGT superfusion of the neonatal rabbit soleus muscle. The toxin treatment resulted in slower synapse elimination, as assessed both physiologically and anatomically, even for muscle fibers whose activity was not completely blocked. While the interpretation of this result is dependent on the possibility that α-BGT has influences other than decreased activity, it appears quite likely that a partial block of postsynaptic activity *can* slow the rate of neuromuscular synapse elimination.

Chapter 3 describes a separate series of experiments in which motor unit twitch tensions were assayed for the soleus muscles of neonatal rabbits. Synapse loss could be assayed separately for fast and slow populations by separating the motor units, based on their twitch rise times. Estimates of the rate of synapse elimination for the two populations suggested that slow muscle fibers were initially more heavily polyinnervated than fast fibers and that they lost synapses at a faster rate, so that both populations of fibers became predominantly singly innervated at about the same time. The remainder of the issues in Chapter 3 are related to the question of whether there are particular attributes of motor neurons that might place the inputs from some neurons at a competitive advantage over others.

The first such issue was whether motor neurons with relatively large axonal arbors are at an advantage or disadvantage in the competition for synaptic sites. If this were the case, it would be expected that the diversity in motor unit sizes would decrease during synapse elimination if a large arbor were a disadvantage, and the diversity would increase if it were an advantage. Contrary to both hypotheses, no significant change in the diversity of motor unit sizes was observed.

The next issue was whether motor neurons from particular positions in the spinal cord were at an advantage or disadvantage compared to the others. To test this issue, mean sizes of motor units from both rostral and caudal extremes of the soleus motor pool were compared to the mean sizes for those from the middle of the pool. At the earliest age tested, when the soleus is still heavily polyinnervated, the motor units from the extremes were no smaller than those from the middle. However, just four days later, the units from each extreme were significantly smaller than those from the middle; this difference persisted in older, singly innervated muscles. There was no significant difference between the rostral and caudal motor units at any age tested. It is concluded that motor neurons from rostral and caudal extremes are at a disadvantage when in competition with those from the middle of the motor pool during synapse elimination in the rabbit soleus.

Finally, a small portion of the rabbit soleus motor neurons was inactivated by implantation of a tetrodotoxin-laden Silastic plug during synapse elimination. Since there was nearly complete overlap between the inactivated and active motor units at the time of the implant, this allowed a test of whether the level of activity of a motor neuron can influence the ability of its terminals to compete for sole occupancy of endplates. It was found that the inactive motor units ended up significantly larger than their active counterparts in normal and control implanted animals, and remained larger even after the endplates were virtually all singly innervated. It is

concluded that inactivity can result in a significant competitive advantage during synapse elimination. The generality of these conclusions and their implications in terms of the ways in which neuromuscular synapse elimination might be regulated are discussed in detail.

CHAPTER 2

Slowing of Synapse Elimination by α-Bungarotoxin Superfusion of the Neonatal Rabbit Soleus Muscle

Abstract

To examine the role of postsynaptic activity in regulating the rate of neuromuscular synapse elimination, contractile activity of neonatal rabbit soleus muscles was decreased by chronic superfusion of α-bungarotoxin (α-BGT) over their surface. Superfusion was begun at 6 days postnatal and continued for a variable duration (2 to 5 days) before muscles were analyzed. The percentage of polyinnervated fibers was assessed both physiologically and anatomically for α-BGT treated muscles and their contralateral muscles, in addition to normal and control muscles of the same age. Within muscles exposed to α-BGT for 5 days, an average of 55% of endplates remained polyinnervated based on either assay. This value was significantly greater than for normal, control-treated, or contralateral muscles of the same age. The anatomical assay further revealed that the retention of polyinnervation in α-BGT-treated muscles was most pronounced near the muscle's surface, although endplates at the center were also affected. This finding, coupled with the observation that only a small percentage of the muscle fibers were completely inactivated, suggests that the activity block was also most pronounced near the surface and relatively low at the muscle's center. The percentage of endplates at which synapse elimination was delayed was greater than the percentage whose activity was completely blocked, suggesting that synapse loss was slowed even in muscle fibers retaining some postsynaptic activity. These observations indicate that the rate of synapse elimination depends on the levels of functional acetylcholine receptors. This process is likely to be mediated in a graded fashion by changes in postsynaptic activity at individual endplates.

Introduction

A number of studies have addressed the influence of activity on the rate of neuromuscular synapse elimination. If activity is increased by chronic electrical stimulation of either the nerve (O'Brien et al., 1978) or both nerve and muscle (Thompson, 1983a), the rate of elimination is increased. Conversely, presynaptic activity blockade induced by tetrodotoxin (Thompson et al., 1979) or botulinum toxin (Brown et al., 1981) slows the rate of elimination. However, none of these experiments clearly addresses the question of whether it is the presynaptic or postsynaptic activity level (or both) that regulates the rate of elimination. Presynaptic activity block also interrupts postsynaptic activity, and each of the electrical stimulation paradigms increase both presynaptic and postsynaptic activity. Duxson (1982) has shown that postsynaptic activity block induced by α-bungarotoxin (α-BGT) halts the normal reduction in number of terminal profiles per endplate observed in electron micrographs of developing neonatal muscle. It is not certain whether this result reflects an increase in complexity of remaining terminals, an actual slowing of the rate of synapse loss, or a combination of both.

In order to determine with greater certainty whether a slowing of synapse elimination can be elicited by a strictly postsynaptic blockade, we continuously superfused α-BGT over rabbit soleus muscle during the normal period of synapse removal. Even though this procedure produced only a partial blockade of the muscle, our assays (both physiological and anatomical) indicate a pronounced and widespread slowing of synapse elimination.

Methods

Experiments were conducted using the soleus muscles of New Zealand White rabbits. The percentage of polyinnervated muscle fibers was assessed both physiologically and anatomically for 3 groups of animals: 1) 16 normal rabbits aged 6 to 11 days postnatal; 2) 13 α-BGT-treated animals, whose left soleus muscles were superfused with α-BGT for a duration of 2 to 5 days, from postnatal day 6 until days 8 to 11; 3) 8 control rabbits treated identically to the α-BGT-treated animals from days 6 to 11, except with sterile Ringer replacing the α-BGT.

Six-day old rabbits were anesthetized with ketamine (120 mg/Kg; intramuscular), and an Alzet osmotic minipump (Alza Corp.) was implanted subcutaneously over the lumbar musculature through an incision at the knee. For α-BGT treatment, the pump contained 0.05 mg/ml of α-BGT (Sigma) dissolved in sterile Ringer (except for 2 animals, for which the dose was 0.06 or 0.075 mg/ml); for control treatment the pump was filled only with sterile Ringer. The pump rate of 1μl per hr and capacity of more than 240μl allowed continuous delivery of the solution for more than 7 days.

The pump contents were delivered to the soleus muscle via a flexible catheter connected to a short length (about 5mm) of polyethylene tubing drawn to a narrow diameter (about 250μm). The catheter was inserted through a small incision in the thinnest and most lateral portion of the lateral gastrocnemius (just over the body of the soleus), so that it was in contact with the soleus at its most lateral edge. The tubing was held in place with a single suture, which closed the muscle incision, and by an additional suture through the bone at the knee. At this point 10μl of α-BGT or control solution were injected via a Hamilton syringe with a 30 gauge needle through the muscle incision and over the surface of the soleus. The injection was

intended as a priming dose, since the pump does not begin to deliver its contents until several hours after implantation. Following closure of the incision through the skin and at least 2 hours for recovery from anesthesia, rabbits were returned to a hutch with their littermates.

Because of the rapid growth of the neonatal rabbits, the catheter often was displaced from the vicinity of the soleus muscle before the terminal experiment. Therefore, only animals in which the catheter tip was clearly in contact with the soleus at the time of dissection were analyzed. Infusion periods exceeding 5 days were not feasible, owing to the low probability of keeping the catheter in place.

For physiological analysis, animals were anesthetized deeply with ether. Both legs were removed and placed in chilled ($\approx 15°$ C.), oxygenated Ringer. The soleus muscles were quickly freed and removed along with several millimeters of the soleus nerve. Less than 10 minutes elapsed between the removal of left and right muscles. One of the muscles was moved to a Sylgard-lined plexiglass recording chamber, where it was continuously superfused with oxygenated Ringer at room temperature; the second muscle was stored for possible later analysis in a Ringer-filled beaker bubbled with oxygen. Generally, both muscles were analyzed physiologically for only the α-BGT-treated animals. The sequence of analysis of the muscles was as follows: tension recording (see below) from the α-BGT treated muscle, tension recording from the contralateral muscle, intracellular recording (see below) from the treated muscle, and intracellular recording from the contralateral muscle. The tension recordings were separated by less than 15 minutes and the initiation of intracellular recording for the 2 muscles by less than 2 hours. It is quite unlikely that the number of detectable inputs per endplate decreased during this period; in our extensive experience with *in vitro* rabbit soleus preparations, we have found that soleus nerve and maximal direct stimulation evoke equal twitch responses for

several hours after dissection, even in singly innervated muscles.

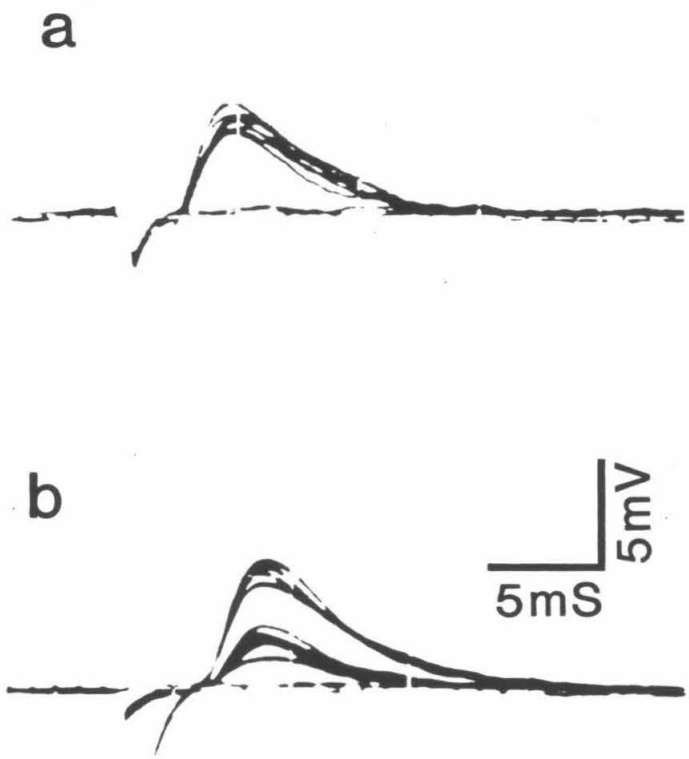
For tension recording, the distal tendon was pinned to the bottom of the Sylgard dish and the proximal tendon attached to a tension transducer via a length of 6-0 silk thread. The muscle was stretched to elicit maximal twitch tension upon direct muscle stimulation, and this response was compared to the twitch response from maximal stimulation of the muscle nerve. In normal and control muscles, the two values were invariably equal (within a few percent attributable to fluctuations in response magnitudes). Therefore, any difference in α-BGT treated muscles (see below) could be used to estimate the percentage of muscle fibers in which functional transmission was blocked.

Muscles were prepared for intracellular recording by cutting the muscle fibers away from the proximal and distal tendons to prevent nerve-evoked contractions (Barstad, 1962). The cut muscle was pinned to the Sylgard, stretching the muscle fibers as tightly as was possible without tearing the preparation. A glass microelectrode (resistance 8-30 MΩ) filled with 3M KCl was used to impale muscle fibers and to record the endplate potential resulting from maximal nerve stimulation. If the endplate potential was greater than about 2 mV, the nerve was stimulated at graded intensity to determine whether the endplate potential arose from multiple inputs (Redfern, 1970; Fig. 1).

Only the first detectable endplate potential in each penetration was assayed in order to avoid the possibility of obtaining artifactual multicomponent potentials that can result from simultaneous recording of two endplate potentials following sequential impalement of the muscle fibers (Soha et al., 1987). For each muscle analyzed, the state of innervation of at least 20 and usually about 30 muscle fibers was determined. All of the recordings were made from fibers at the anterior side

Figure 1. Endplate potentials recorded from singly innervated (a) and multiply innervated muscle fibers (b) following graded stimulation of the muscle nerve. The muscle fibers were cut to lower their resting potential so that the resultant increase in Na^+ channel inactivation would prevent stimulus-evoked muscle contraction. For panel (a), the smallest response that could be evoked was identical to that from supramaximal nerve stimulation, confirming that the response was recorded from a singly innervated endplate. For panel (b), the response measured was of 2 discrete magnitudes, the smaller response being evoked at a lower stimulus intensity than the larger response, which is the potential evoked by simultaneous activation of the first and a second terminal by supramaximal nerve stimulation.

FIGURE 1



of the muscle. For normal, control-treated, and contralateral to α-BGT-treated muscles, endplates were sampled without regard for their proximo-distal or medio-lateral position in the muscle. However, for the toxin-treated muscles, the catheter tip invariably contacted the muscle at its lateral edge and about midway along its length; it was most difficult to record endplate potentials in this region, presumably because of more extensive postsynaptic activity block. Therefore, in these muscles the endplate sample was biased away from this region.

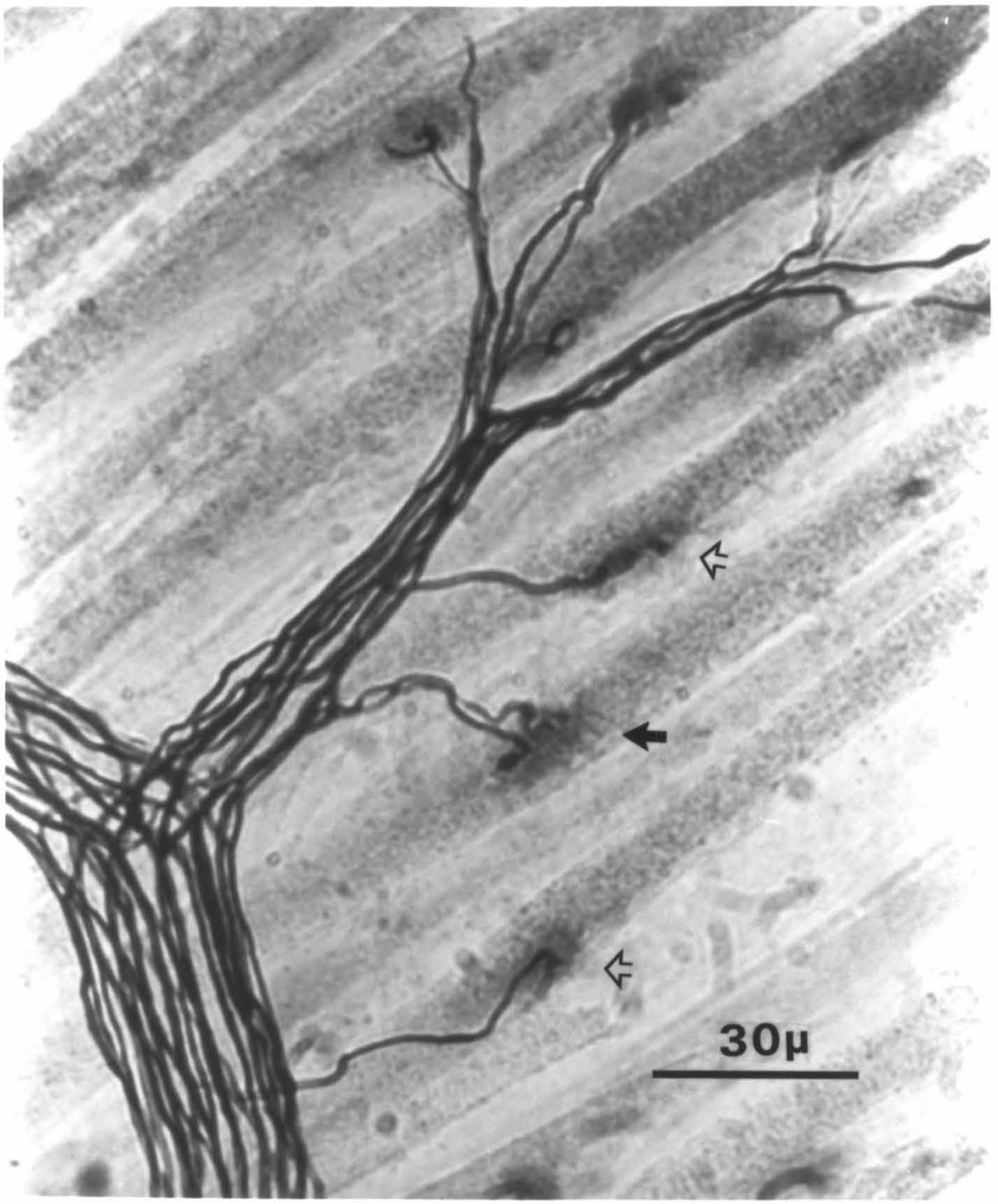
Following physiological analysis, muscles were pinned out in a small Sylgard-lined petri dish and processed for silver staining of nerve terminals to allow subsequent anatomical analysis of the degree of polyinnervation. Even if physiology was done on only one muscle from an animal, the contralateral muscle was also stained. A variation of the silver-cholinesterase method described by Hopkins et al. (1985) was used. Muscles were fixed and stored in buffer solution per their description, then cut longitudinally into 40μm sections with a freezing microtome. Sections were mounted on gelatin-subbed slides, which were then placed in chilled Karnovsky cholinesterase stain (Karnovsky and Roots, 1964) with no substrate for 6-7 minutes, followed by a 5-minute water rinse. Endplate sites were stained for esterase for 15-20 minutes at 37° C, as described by Pestronk and Drachman (1978), producing a transparent blue reaction product through which the nerve terminals could be visualized. Sections were subsequently processed as described by Hopkins et al. (1985).

Endplates from each muscle were observed at 400X magnification to determine the number of contributing axons. Endplate-rich regions of muscle sections were identified, and every endplate in the field of view, as defined by presence of esterase staining, was examined. Only complete endplates not intersecting the plane of section were characterized. After observing all the endplates in the field, the stage

was moved to bring more endplates into view, all of which were then characterized. Typical singly innervated and polyinnervated endplates are shown in Figure 2.

Preliminary analysis suggested that α-BGT-treated muscles might have more polyinnervated endplates at the periphery than at the center. Therefore, for α-BGT-treated muscles, control-treated muscles, and the muscles contralateral to them, endplates within about 8 fiber diameters (ca. 70-120μm, depending on the muscle's size and age) of the section's edge were designated as peripheral fibers and were scored separately from those in the center. Assuming a cylindrical muscle with 11,000 fibers (Bixby and Van Essen, 1979), the radius would be 59 fibers. Thus, the percentage of fibers corresponding to the peripheral region would be about 23%. Sections were cut parallel to the anterior face of the muscle; thus, their periphery corresponded to the lateral and medial borders between which we could not distinguish. Only sections cut after progressing deeply enough into the muscle to insure an adequate representation of both the center and the periphery were stained. At least 30 endplates from each region were characterized for each muscle, and the slides were coded for unbiased analysis. Normal muscles were analyzed separately but were also coded. For these muscles, at least 100 endplates were examined per muscle, and because there was no indication of a difference in degree of polyinnervation at the center versus the periphery in control or contralateral muscles, the location of endplates was not considered.

Figure 2. Photograph of a silver-cholinesterase-stained soleus muscle section from a normal, 11-day-old rabbit. The majority of the endplates are singly innervated (open arrows), while only one endplate in the photograph is multiply innervated (two inputs, solid arrow). Scale bar = 30μm.


30µ

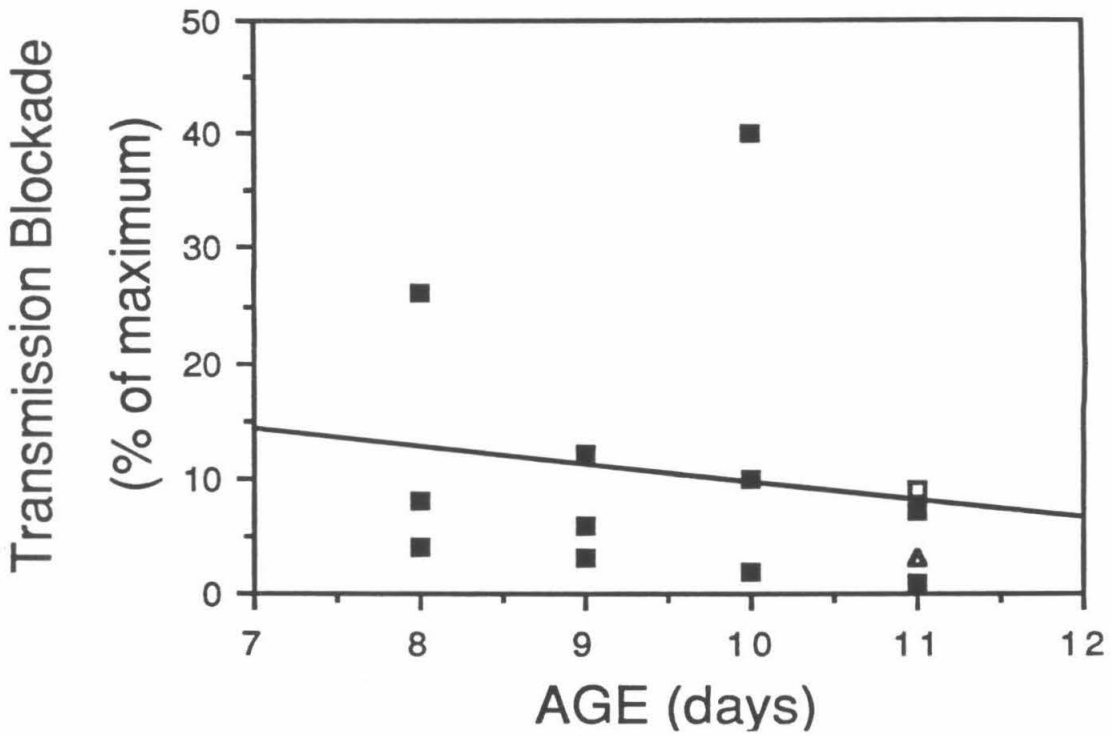
Results

In all muscles treated with α-BGT, there were vigorous, nerve-evoked contractions immediately upon testing the dissected preparation, indicating that transmission blockade was not complete. To estimate the extent of the functional blockade produced by the toxin superfusion, we compared the maximal twitch responses from nerve-evoked and direct muscle stimulation. Figure 3 plots the measured degree of transmission blockade for the 13 toxin-treated muscles according to their age (and duration of treatment), following initiation of treatment at day 6. In this figure, the difference between direct and nerve-evoked responses is expressed as the percentage of the direct response. In general, the degree of block is quite low, exceeding 15% in only 2 cases. For untreated muscles, there was no indication of a significant functional blockade (maximum of 3% difference for normal muscles, control muscles and those contralateral to toxin treated muscles).

There was a slight tendency for the percentage of fibers blocked in toxin-treated muscles to decline with age. The least-squares regression line for the points in Fig. 3 (shown by the line) has a slope of negative 1.6, corresponding to a decrease of 1.6% per day, but this slope is not significantly different from zero. Nevertheless, there is other evidence suggesting that the effectiveness of the toxin block decreased with age. It was generally very difficult to record endplate potentials from the most superficial muscle fibers of 8-9 day, α-BGT-treated muscles; usually, the first measurable endplate potentials were encountered only after penetrating several layers of muscle fibers. This was seldom the case for the 10-11 day, α-BGT-treated muscles and never for normal or control muscles. There are several factors that might be expected to contribute to the change with age. There might be poorer toxin penetration of the muscle as its diamater grows, or blockage of toxin delivery as connective tissue accumulates near the catheter end; alternatively, the pump rate

Figure 3. The percentage of fibers that could not be activated by nerve stimulation, plotted against age of soleus muscles treated with α-BGT from day 6 postnatal. Each filled square represents the percentage of fibers blocked by α-BGT treatment at the time of the assay for a single muscle. The best linear fit to the values is indicated by the diagonal line and indicates a slight tendency for the percentage of blocked fibers to decline with age. The slope of the line is -1.6, corresponding to a decrease of 1.6% per day. All of the muscles were treated with α-BGT at a concentration of 0.05 mg/ml except for one muscle treated with 0.06 mg/ml (open triangle) and another with 0.075 mg/ml (open square).

FIGURE 3


Transmission Blockade
(% of maximum)
50
40
30
20
10
0
7
8
9
10
11
12
AGE (days)

might not have been suitable to maintain the initial degree of blockade caused by the priming dose.

The need to penetrate through several fibers to obtain adequate endplate potentials in the younger muscles strongly suggests that there was more activity block at the periphery than centrally. A positional asymmetry in endplate potential magnitude was also apparent at the later ages. In this case, endplate potentials were easily detected at the medial side but less frequently on the lateral side, closer to the catheter tip. Taken together, these observations argue that the degree of activity *blockade* was generally greater at the periphery than centrally, and also with a bias toward more blockade at positions closer to the catheter tip.

The need for a minimum endplate potential size for physiological assay presumably biased our sample toward less blocked endplates. Coupled with the low incidence of complete endplate activity blockade, this suggests that suprathreshold endplate potentials occurred at many of the physiologically assayed endplates. Nevertheless, the degree of polyinnervation at the periphery of toxin-treated muscles was nearly identical, whether based on the physiological or anatomical assay.

The two muscles with slightly higher doses of α-BGT (Fig. 3, open symbols) did not appear to be significantly better blocked than the others. Animals treated with doses higher than 0.075 mg/ml α-BGT seldom survived more than 2 days after implantation and those that did gained little or no weight. None of these animals was assayed.

Despite the incomplete nature of the activity blockade, the α-BGT treatment markedly slowed the rate of synapse removal. We will first consider the results from the full five-day treatment, starting at day 6 and ending at day 11, where the effect was most dramatic. These comparisons allowed us to address separately the issues

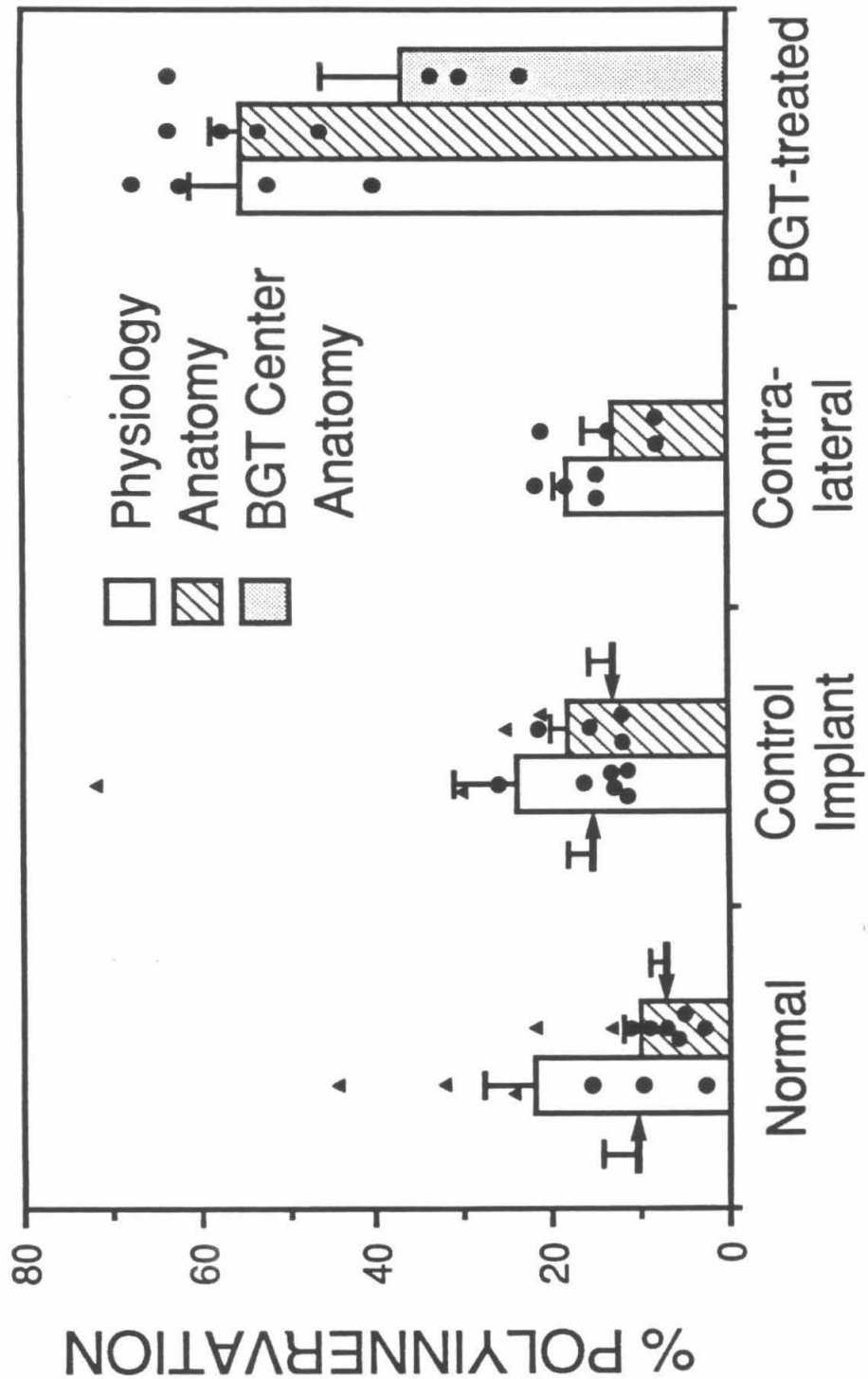
of whether the overall procedure had any significant effect and to what degree the effect was attributable to specific versus non-specific factors. Figure 4 summarizes the physiological and anatomical results, as mean percentage-polyinnervation values ($\pm$ S.E.M.), for the toxin-treated muscles and the various control groups (normal, control implant, and contralateral to toxin-treated); the values for individual muscles are also plotted for each group. There are several important comparisons to be made among these groups.

First, consider the incidence of polyinnervation for toxin-treated muscles versus the contralateral muscles of the same animals. The 4 toxin-treated muscles averaged $55 \pm 6\%$ polyinnervation based on the physiological assay; estimated polyinnervation based on the anatomical assay was $55 \pm 4\%$ for peripheral and $37 \pm 9\%$ for central fibers. Each of these values was significantly greater than the values from the 4 contralateral muscles, whose percentage polyinnervation averaged $18 \pm 2\%$, based on physiology, and $13 \pm 3\%$ based on anatomy (central and peripheral fibers combined, see Methods; $p << 0.001$ based on physiology or anatomy at the periphery and $p < 0.005$ at central fibers, Student's T-test). In addition to the larger values for toxin-treated muscles than their contralateral muscles, at both the periphery and centrally, the difference between the peripheral and central fibers within the toxin-treated muscles was significant ($p < 0.02$). These results imply that there was a marked effect of the toxin treatment both peripherally and centrally, and that the effect was significantly more pronounced at the periphery.

Next, consider the comparison between toxin-treated and control-implanted muscles. Since these control muscles were treated identically to the toxin-treated muscles, except for the presence of toxin in the superfused solution, it is useful to distinguish between these and other "control" muscles (normal and contralateral to toxin-treated); this comparison tests whether the effects in α-BGT-treated muscles

Figure 4. Bar graph of the mean ± standard error of the mean, levels of polyinnervation for normal, control-treated, α-BGT-treated, and contralateral to α-BGT-treated soleus muscles, all analyzed at 11 days postnatal. Control and α-BGT treatments were initiated at 6 days postnatal. Open bars represent the means of the values from each group of muscles based on physiological assessment of the percentage of polyinnervated endplates; hatched bars represent mean values based on anatomical assessment of peripheral endplates for α-BGT-treated muscles or of the average for central and peripheral fibers for other groups; and the value for the stippled bar is based on anatomical assessment at central endplates of α-BGT-treated muscles. The mean values for α-BGT-treated muscles are significantly larger (both central and peripheral endplates) than those from either normal, control-treated, or contralateral muscles, based on either physiological or anatomical assays. The value for peripheral endplates is also significantly greater than that for central endplates. See text for mean values and degree of significance. The values obtained for individual muscles are indicated by filled circles on (or above) the appropriate bars. Values indicated by triangles are from animals from developmentally immature litters (see text). The means ± S.E.M. for normal and control-treated muscles when values indicated by triangles are excluded are indicated by the levels of the arrows and by the error bars beside appropriate bars. These means and standard errors are: for normal muscles, 10 ± 4% based on physiology (3 muscles) and 7 ± 2% based on anatomy (6 muscles); for control-treated muscles, 15±2% based on physiology (6 muscles) and 13±2% based on anatomy (4 muscles).

FIGURE 4


% POLYINNERVATION
80
60
40
20
0
Physiology
Anatomy
BGT Center
Anatomy
Normal
Control
Implant
Contra-
lateral
BGT-treated

are specifically attributable to the toxin treatment. Eight control-implanted muscles were analyzed physiologically, of which six were also analyzed anatomically. The mean percentage polyinnervation from the control-treated muscles was $24 \pm 7\%$, based on physiology and $18 \pm 2\%$ based on anatomy. The p values from Student's T-test for the control versus toxin-treated comparisons were $p < 0.001$ for comparisons based on peripheral fibers from the toxin-treated muscles (anatomical or physiological assay) and $p < 0.005$ based on central fibers. We therefore conclude that there was a large effect specifically attributable to the toxin treatment.

Assessment of non-specific effects. In addition to this specific effect of α-BGT in the toxin treated muscles, there might have been a smaller non-specific effect of the treatment as well, relating to local trauma around the implantation site, a systemic effect of the toxin, or general stress from the implantation procedure. To assess these possibilities, we looked for differences among the three control groups (normal, control-implanted, and contralateral to toxin treated muscles). Mean $\pm$ S.E.M. values for control-implanted and contralateral to toxin-treated muscles have been shown; the values for normal muscles were $22 \pm 6\%$ based on physiology and $10 \pm 2\%$ based on anatomy. There is a statistically significant difference in only one of the pairwise comparisons within these three groups, the control versus normal comparison based on the anatomical assay. On the other hand, there was considerable variability in the values for individual muscles, and in particular one of the control-implanted muscles had a very high degree of polyinnervation (72%; 18/25), based on the physiological assay. These observations raise the possibility that there was a non-specific effect in at least some muscles.

In order to assess this possibility more thoroughly, we examined the contralateral muscles in the two cases of control implants with the highest degree of polyinnervation (72% and 30% assayed physiologically; triangles in Fig. 4), and

we also examined one or two muscles from normal animals belonging to the same litter. The rationale for this is that the variability in the degree of polyinnervation is known to be less within a litter than between animals from different litters. This analysis revealed that the measured level of polyinnervation was relatively high in all of the comparison muscles. For the two muscles contralateral to the control implant, polyinnervation was 60% for each in the physiological assay and 25% and 30% for the anatomical assay (data not shown). For the three muscles taken from the same litters, polyinnervation averaged 33% assayed physiologically and 18% assayed anatomically (individual values shown by triangles in Fig. 4).

It should be noted that the overall mean values from the normal animals are probably biased toward a somewhat high percentage of polyinnervated fibers, since half of the muscles assayed physiologically (3/6) and a quarter of those assayed anatomically (2/8) were not selected at random, but only after a difference was suspected for their litter. The difference in these proportions probably accounts for some of the difference between the physiologically and anatomically determined means for normal animals; and it might also account for the lack of a significant difference between control-implanted and normal muscles based on the physiological assay.

We conclude from this that there was an unusually high degree of polyinnervation in both normal and control-implanted animals from two of the litters used in this study. It remains uncertain whether the difference between normal and control animals reflects a significant non-specific effect buried within this developmental noise. However, the lack of a difference for the other comparisons argues against some of the possible types of non-specific effects. For example, an effect due to local trauma should influence only the treated muscle in control-implanted animals, not their contralateral muscles; but there was no significant

difference *between* these groups (data not shown). Also, if there were a systemic effect of the toxin, there should be a difference between control-implanted and contralateral to toxin-treated muscles (assuming no local trauma, as argued above). Thus, if there is a non-specific effect, it is probably related to general stress from the implantation procedure – the normal muscles were the only group that might have differed and were also the only group not subjected to this potential effect. At any rate, if there is such an effect, it is not nearly as large as the effect specifically attributable to toxin treatment.

Rate of synapse elimination. In order to determine the time course of the toxin effect, we measured the incidence of polyinnervation in toxin-treated, contralateral, and normal muscles at successive days from day 8 to day 11, i.e., 2-5 days after the implantation. Results from normal animals are shown in Figs. 5a and 5b, for physiological and anatomical assays, respectively. To avoid the possibility of bias due to non-random sampling in the 11-day old sample (see above), the values from unusual litters, indicated by triangles in Fig. 4, were not included for this part of the analysis. As expected, there was a steady decline in the percentage of polyinnervated fibers with age. The physiological values declined from 73% at day 6 to 10% at day 11 (a rate of 13% per day) and the values from silver stains from 66% to 7% (12% per day).

Figure 6 shows that the α-BGT-treated muscles had lost measurably fewer synapses than their contralateral muscles as early as 2 days after the the implant and that they continued to lose synapses, but at a substantially decreased rate throughout the duration of the treatment. The percentage of polyinnervated fibers assessed physiologically and plotted against age from α-BGT-treated and contralateral muscles is shown in Fig. 6a. For each treated muscle (filled squares), the value for the contralateral muscle from the same animal is shown directly below

Figure 5. The percentage of polyinnervated endplates plotted against postnatal age for individual normal muscles based on the physiological assay (a) and for the same muscles plus the contralateral muscles from the same animals, based on the anatomical assay (except for one muscle not stained at day 10, b). The best linear fits to the values are indicated by the diagonal lines. Based on either assay, the percentage of polyinnervated endplates declines dramatically with age, reflecting the elimination of redundant synapses. The values based on the physiological and anatomical assay are similar at every age.

FIGURE 5

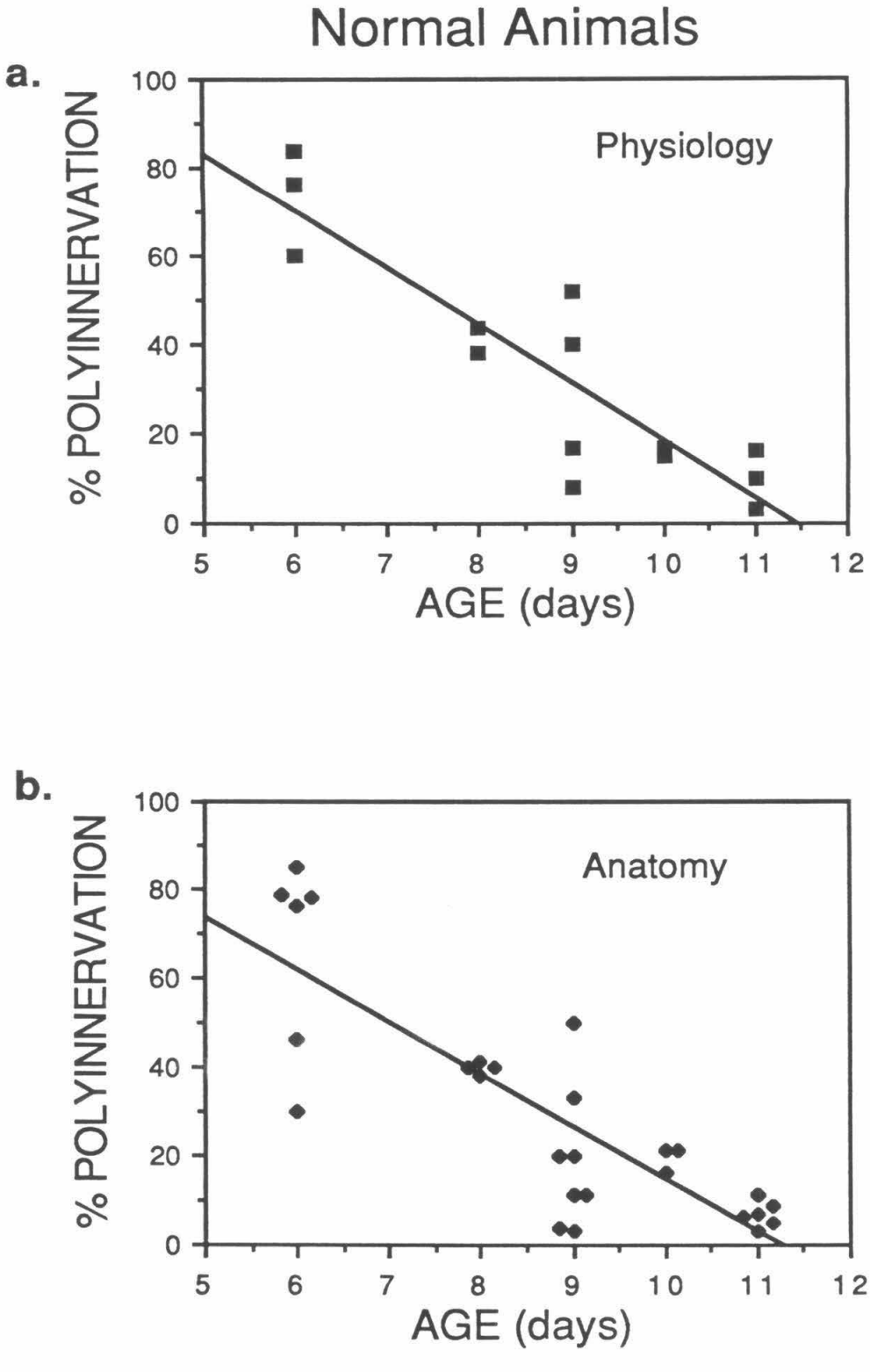
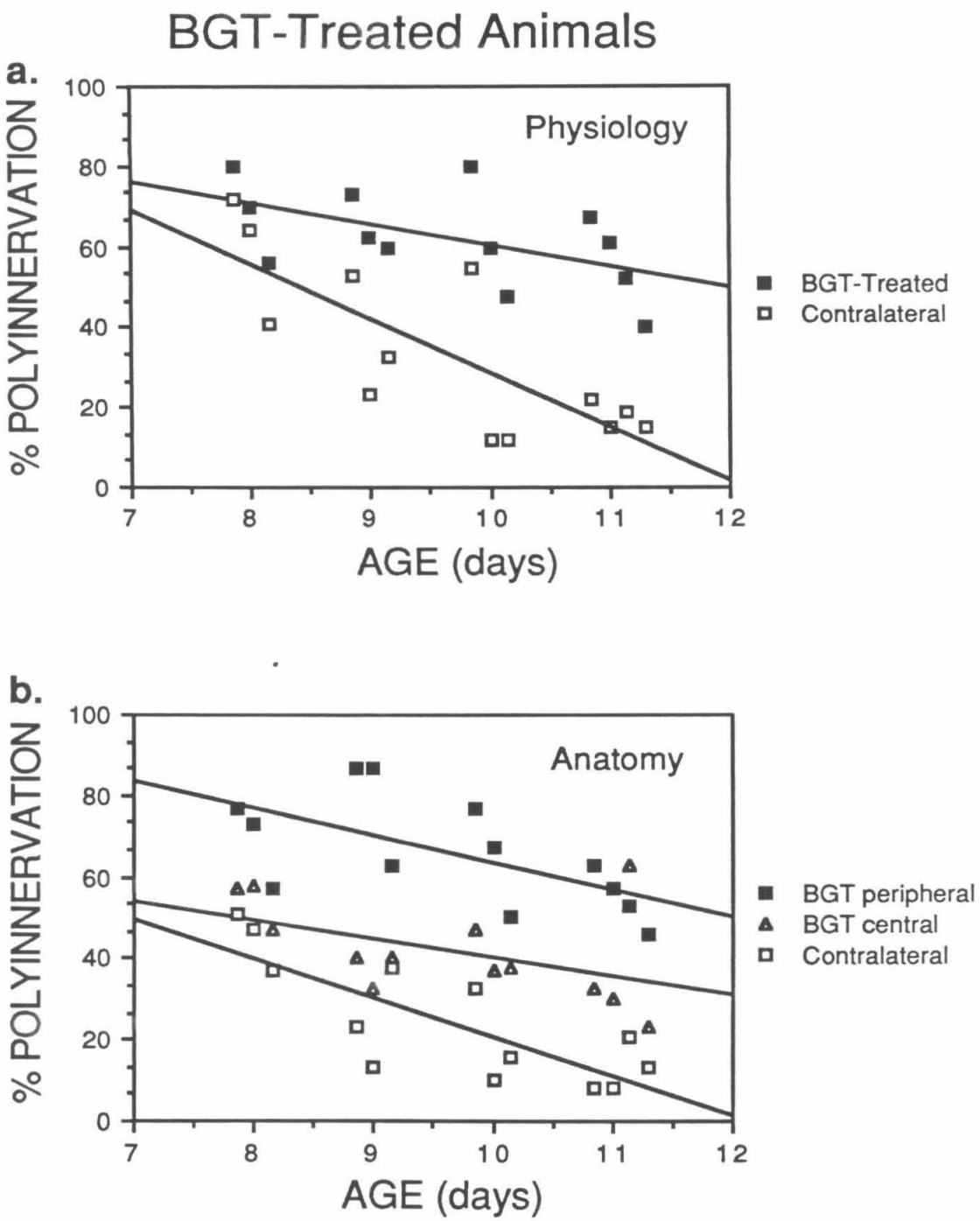

Normal Animals
a.
% POLYINNERVATION
100
80
60
40
20
0
Physiology
5
6
7
8
9
10
11
12
AGE (days)
b.
% POLYINNERVATION
100
80
60
40
20
0
Anatomy
5
6
7
8
9
10
11
12
AGE (days)

Figure 6. The percentage of polyinnervated endplates plotted against age for individual muscles from α-BGT-treated animals. Physiologically determined values are plotted in (a) and anatomically determined values in (b). For both (a) and (b) the values from the α-BGT-treated muscles are indicated by filled squares with the value for the contralateral muscle from the same animal indicated by an open square directly below. In (b), the values for peripheral and central endplates from the treated muscles are indicated by filled squares and open triangles, respectively. The best linear fits to the values from each group are shown by the diagonal lines. The rate of elimination is similar to normal at endplates from contralateral muscles, while it is substantially slower than normal for endplates from both the center and periphery of α-BGT-treated muscles. The single 11-day muscle with a larger estimated percentage of polyinnervation at the central than peripheral fibers corresponds to the animal that was treated with a slightly higher dosage of α-BGT (0.075 mg/ml, open square of Fig. 3).

FIGURE 6


BGT-Treated Animals
a.
% POLYINNERVATION
100
80
60
40
20
0
7
8
9
10
11
12
AGE (days)
Physiology
BGT-Treated
Contralateral
b.
% POLYINNERVATION
100
80
60
40
20
0
7
8
9
10
11
12
AGE (days)
Anatomy
BGT peripheral
BGT central
Contralateral

(open squares). For every animal, the degree of polyinnervation was greater in the treated muscle than in the contralateral muscle. Based on physiological analysis, the percentage polyinnervation in α-BGT-treated muscles declined at a rate of only 4% per day from day 6 (73% polyinnervation from normal animals) to day 11 (55% polyinnervation). For the contralateral muscles, the rate of elimination was comparable to that for normal muscles (11% per day for contralateral versus 13% for normal).

Results based on silver stains are plotted in Fig. 6b; in addition, fibers from the periphery and the center of the treated muscles are considered separately (indicated by filled squares and open triangles, respectively). For every animal, the degree of polyinnervation was greater for both the peripheral and the central fibers of the treated muscle than for the contralateral muscle. The peripheral fibers were more heavily polyinnervated than the central fibers in all but one of the α-BGT-treated muscles. The rates of elimination based on these values from day 6 to day 11 (calculated from 66% polyinnervation at day 6) were 2% per day for peripheral and 6% for central fibers from α-BGT-treated muscles, and 11% for the contralateral muscles versus 12% for normal.

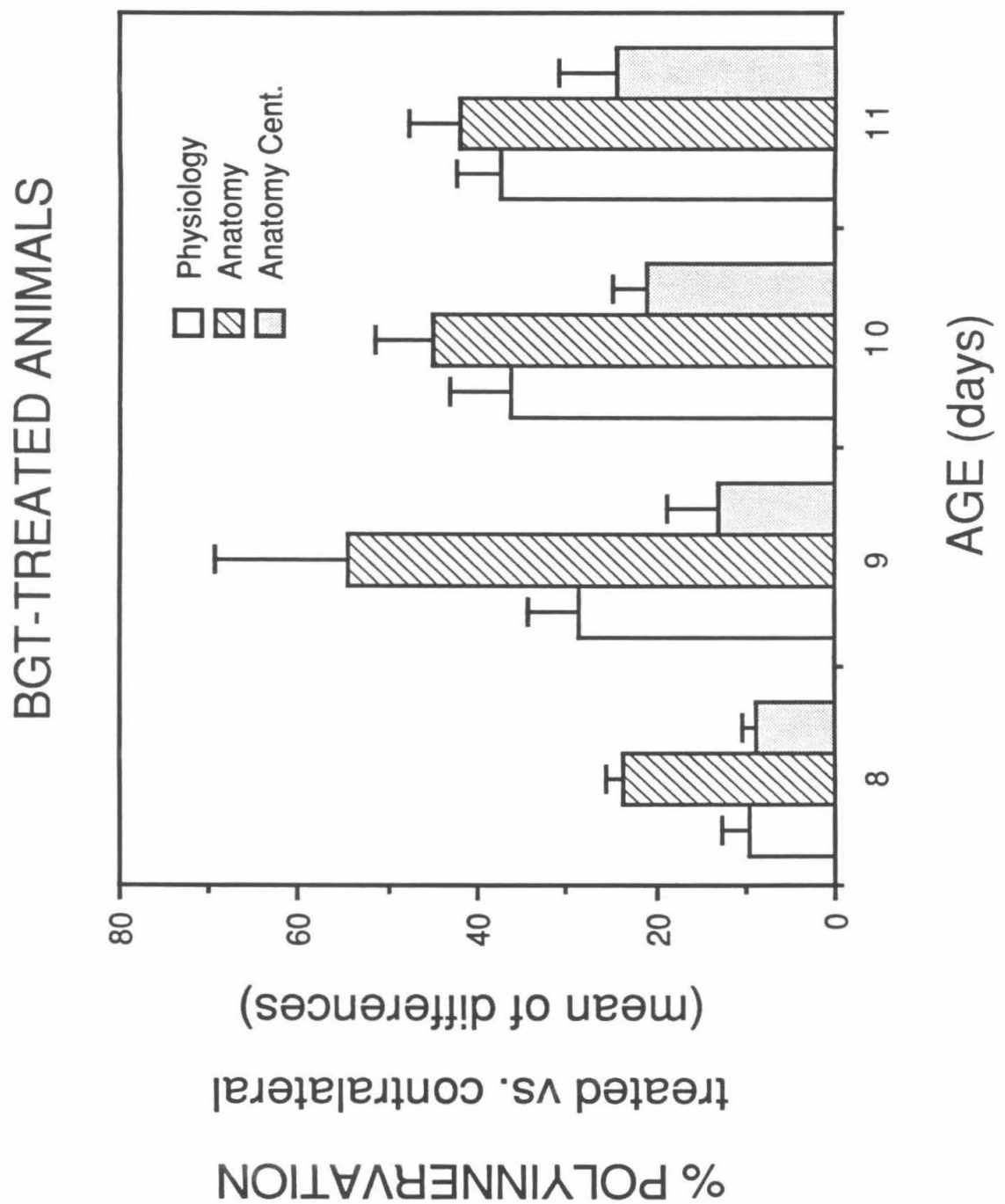
In order to obtain a more sensitive measure of these changes with time, we calculated the difference in polyinnervation between the treated muscle and the paired contralateral muscle for each toxin-treated animal. This comparison is not sensitive to the substantial variations between animals that is apparent even in normal muscles (cf. Fig. 5); it also controls for the possibility of small, non-specific implantation effects suggested by the analysis above. Figure 7 shows the mean ($\pm$ S.E.M.) of this differential measure at each age. Values based on physiology and on anatomy at the periphery and centrally are indicated separately. The tendency for the differences to increase over time may reflect the continued differential loss

of synapses between α-BGT-treated and their contralateral muscles. However, this difference might be attributable to a saturation effect, since neither of our assays distinguished between fibers with 2 inputs and those with 3 or more inputs, which might have been especially common at day 8. All of the mean values shown in Fig. 7 are significantly larger than zero ($p < 0.05$, Student's T-test on mean of differences versus zero) except for the value from central fibers at day 9. This indicates that there was a significant effect as early as 2 days after initiation of the toxin treatment.

The overall significance of the comparisons can be further tested, based on pairwise comparisons between the values obtained from individual animals by the same assay using the sign test. Based on either assay at either the periphery or centrally, the percentage polyinnervation was greater for the treated than for the contralateral muscle in all 13 cases; this is significant at the level, $p < 0.001$. Based on anatomy, central fibers were more heavily polyinnervated than peripheral fibers in 12 of 13 cases, which is significant at the level, $p = 0.002$.

Figure 7. The difference in percentage of polyinnervated endplates between treated and contralateral muscles from α-BGT-treated animals. Values shown by bars are the mean $\pm$ S.E.M. of the values from the individual animals at each age. Values based on physiology are shown by open bars, anatomy at the periphery by hatched bars, and anatomy at the center by stippled bars. The differences for all 3 groups increase with age (duration of treatment).

FIGURE 7


BGT-TREATED ANIMALS
Physiology
Anatomy
Anatomy Cent.
% POLYINNERVATION
treated vs. contralateral
(mean of differences)
0
20
40
60
80
8
9
10
11
AGE (days)

Discussion

These experiments were originally intended to determine whether synapse elimination can be slowed during an activity blockade imposed by a postsynaptic blocking agent. Our interpretation with regard to that particular issue is affirmative, in agreement with Duxson (1982). The interpretation becomes particularly intriguing, in our opinion, in light of evidence that the receptor blockade was far from complete. These observations have several implications in terms of the mechanisms by which competition between synapses for sole occupancy of endplates may occur.

Does postsynaptic receptor block slow synapse elimination? Most, if not all, of the effect observed for toxin-treated muscles was specific and was restricted to the treated muscle. Endplates from the treated muscles retained significantly more synapses than control treated, normal, or contralateral muscles, based on both physiological and anatomical assays. Furthermore, the differences in the rate of elimination were not small, but rather were two- to fivefold. Although there may have been minor effects of control implants, they were quite small relative to the effects of α-BGT treatment.

It is unlikely that decreased postsynaptic activity indirectly caused slower synapse elimination by reducing presynaptic activity by way of reflex circuitry, since deafferentation does not slow synapse elimination (Caldwell and Ridge, 1983). If presynaptic acetylcholine receptors (AChRs) were present at neuromuscular terminals, α-BGT might reduce presynaptic activity directly. However, recent investigations by electron microscope autoradiography designed to detect low levels of presynaptic α-BGT binding failed to demonstrate levels significantly greater than zero (Jones and Salpeter, 1983). Similarly, Lindstrom et al. (1983) observed

no presynaptic binding of antibodies to AChRs located by colloidal gold (see also Jones, 1987, for review). It is a formal possibility that the α-BGT effect was not due a specific postsynaptic blockade, but rather was due to some as yet unknown side effect of the toxin. This possibility seems unlikely; that the effect of α-BGT on neuromuscular transmission and on synapse elimination were both most pronounced at the muscle's periphery further argues against it. We conclude that the toxin treatment slowed the rate of neuromuscular synapse elimination by way of a direct effect on postsynaptic AChRs.

Do presynaptic terminals compete for stabilization by postsynaptic acetylcholine receptors? It has been suggested by Pestronk and Drachman (1985) that sprouting in adult muscle is mediated by interactions between neuronal elements and extrajunctional AChRs that appear when muscle is inactivated. In support of this hypothesis, they reported that α-BGT inhibits sprouting that occurs following inactivation by botulinum toxin. Because a complete AChR blockade was necessary to inhibit sprouting, they argued that only small numbers of functional AChRs are necessary to mediate sprouting. An obvious corollary to this hypothesis is that AChRs might also play an important role in the stabilization of nerve terminals. For example, during synapse elimination, the multiple terminals at an endplate might compete directly for access to a stabilizing influence provided by AChRs, which is distinct from any effects of suprathreshold muscle activity.

Intuitively, it might seem likely that competition would proceed more rapidly when there is a reduced level of a factor that provides positive feedback. Indeed, computer simulations of neuromuscular synaptic competition have confirmed that the rate of synapse elimination can increase upon reduction of the level of a postsynaptic factor ("scaffold") that acts to stabilize nerve terminals (Soha et al., in preparation). However, this is not the only possible outcome in situations of

this type. In colloquial terms, struggling over crumbs can sometimes take a long time. In the context of the receptor hypothesis, suppose that AChRs enhance the dynamic state of nerve terminals by increasing the tendency to withdraw as well as to sprout. In this manner, a decrease in AChRs could conceivably slow the rate of elimination by decreasing the frequency with which any changes take place. Such a hypothesis, while unlikely in our opinion, cannot be ruled out by the evidence currently available.

Is the rate of synapse elimination sensitive to graded changes in postsynaptic activity? If one considers only the observation that synapse elimination slows when activity (either pre- or postsynaptic) is completely blocked (Thompson et al., 1979; Brown et al., 1981; Duxson, 1982), it does not strictly follow that activity normally plays any role in the regulation of the rate of synapse elimination. Since all muscle fibers are presumably active throughout the period of normal synapse loss, these experimental results could be interpreted as a pathological reaction to complete inactivity. Experiments in which the rate of synapse elimination was increased by chronic stimulation, however, indicate that the rate of elimination can indeed be sensitive to graded changes in activity (O'Brien et al., 1978; Thompson, 1983a). Recall, though, that these experiments do not distinguish between effects related to changes in pre- versus postsynaptic activity. It is therefore important to assess to what degree the present results argue for postsynaptic effects associated with graded changes in activity.

The first issue is whether synapse elimination was slowed in fibers that retained a significant level of contractile (suprathreshold) activity. The following semi-quantitative argument suggests that this is indeed the case. In 5 of 6 muscles analyzed 2 to 3 days after initiation of α-BGT treatment, fewer than 15% of the muscle fibers were functionally inactive in our *in vitro* assay. However, the relation

between our assay and *in vivo* conditions is not necessarily direct. While all of the inputs to an endplate were stimulated simultaneously in the assay, they are presumably not simultaneously active at all times *in vivo*. If some of the inputs were subthreshold, this would imply that the percentage of inactive fibers *in vivo* would be somewhat greater than *in vitro*. But this might be offset by decreased transmission at the lower temperatures *in vitro* or by facilitiation following repetitive firing *in vivo*. In any event, the average percentage of blocked fibers based on the *in vitro* tension assay was about 10%; we consider it unlikely that the average exceeded 20% *in vivo* and extremely unlikely that this percentage exceeded 40%.

With these values in mind, we now estimate the percentage of fibers that were actually affected by the α-BGT treatment. Based on the physiological analysis, polyinnervation averaged 73% at day 6 and declined to 55% in toxin-treated muscles by day 11 and to 18% in the contralateral muscles. Thus, 55% of the fibers (73% minus 18%) were converted to single innervation in the contralateral control muscles, but only a third as many (18%) in the toxin-treated muscles. In other words, synapse elimination was delayed on two-thirds (67%) of the fibers that would normally have become singly innervated. Similarly calculated values based on anatomy are 79% for peripheral fibers and 45% for central fibers. There are some uncertanties in these estimates, but they strongly suggest that more than half of the fibers in the entire muscle were affected by the toxin treatment. Since this value is much greater than any of our estimated upper limits on the percentage of completely blocked fibers, it follows that the toxin treatment must have slowed synapse elimination in many fibers whose activity was not completely blocked.

Given that postsynaptic receptor block slowed synapse elimination, even at fibers that were not completely inactivated, we favor the straightforward interpretation, that decreased postsynaptic activity slowed the rate of elimination in a graded

fashion. We have already considered and argued against one alternative possibility, namely, that the slowing of elimination was not related to the change in activity, but rather to a direct influence of AChRs on the stability of presynaptic terminals. Another alternative is that the persistence of multiple innervation on active muscle fibers was an indirect consequence of the complete activity block that did occur at a minority of the muscle fibers. For example, diffusion of sprouting factor from completely inactive fibers could delay synapse loss for all fibers, including active ones. It is relevant in this regard that partial blockade of both presynaptic and postsynaptic activity apparently delays synapse elimination for some of the normally active motor units (Chapter 3). A diffusible influence provides one possible explanation for these results. On the other hand, the Chapter 3 results can also be explained by sensitivity of individual endplates to graded activity changes; and in the present study, diffusion would need to occur over rather long distances (perhaps several hundred μm) to account for the large effect at the central fibers, which were quite far from the majority of the inactivated fibers at the periphery. (The diameter of the rabbit soleus is ca. 3 mm at this age.) If the ability of this presumed factor to have effects at a distance were similar to the ability of inactive muscle fibers to induce sprouting from adjacent terminals, it would be limited to a distance of a few muscle fibers at most, probably not enough to account for the effects we observed. Furthermore, in some muscles, terminals can be induced to sprout only by inactivation of the muscle fiber they innervate (Brown et al., 1980).

Relevance to differential activity effects. We have reported an effect of differential presynaptic activity on the competitive ability of a neuron's terminals, in which inactivity provides an advantage (Callaway et al., 1987; Chapter 3). It is interesting to consider whether there is a single hypothesis that can account for both that result and the present observations. Suppose that there is a trophic substance

that supports terminal maintainance and growth, whose production is regulated by the level of postsynaptic activity, as proposed by Thompson (1985). The present results could be explained if each muscle fiber produced the substance at a rate dependent on its activity level, with less activity resulting in greater production of the trophic substance. But could this also explain an advantage to the terminals of less active motor neurons? This would indeed be the case if the substance could be taken up only by the nerve terminals innervating the muscle fiber where it was produced, and if such uptake increased the competitive ability of all the terminals of the motor neuron that acquired it.

To illustrate this point, consider two different motor units, one with much lower activity than the other. Muscle fibers within each motor unit would initially be polyinnervated, typically by a mixture of low- and high-activity inputs. The overall level of activity would on average be lower for the fibers belonging to the less active motor unit. These fibers would therefore produce more trophic factor. Since each muscle fiber's activity level is normally determined by the activity of the neurons contributing to its innervation, the activity of muscle fibers innervated by less active motor neurons would (averaged over hundreds of fibers in a motor unit) be relatively low. Thus, averaged over all of its terminals, a relatively less active motor neuron would have access to more of the proposed trophic substance. This increased access to trophic substance would place the terminals of the less active motor neuron at an advantage in competition with terminals from the more active neuron. It is important to note, however, that this is not the only mechanism by which less activity could be advantageous; other mechanisms, including some that invoke a dependence on presynaptic activity levels, have been considered (Chapter 3).

Another possible mechanism by which local, graded changes in postsynaptic activity could regulate the rate of synapse loss is suggested by the observation

that low Ca^{++} levels and inhibition of protease activity can slow the rate of synapse elimination (O'Brien et al., 1984; Connald et al., 1986). O'Brien et al. (1984) propose that a Ca^{++}-activated neutral protease actively decreases terminal stability during synapse elimination and that the dependence of synapse loss on activity is related to higher Ca^{++} levels (and therefore to greater protease activity) associated with increased activity. For proteases to account for the present findings, a dependence on postsynaptic activity would be required, and the relevant protease would probably be localized to muscle, where such proteases have, in fact, been demonstrated (O'Brien et al., 1978).

Overall, we conclude that postsynaptic activity is probably an important factor in the regulation of synapse elimination rate. This would imply that the dependence of synapse elimination rate on activity levels is at least in part mediated by a postsynaptic mechanism; such a mechanism could be related to a trophic factor, protease, or both. But even though dependence on a trophic substance is eminently plausible, there is no direct evidence that such a substance exists in neonatal muscle. Proteases, on the other hand, have been directly implicated, and their presence in both muscle (O'Brien et al., 1978) and nerve (Schlaepfer and Hasler, 1979) makes them a strong candidate for both presynaptic and postsynaptic, activity-dependent regulation of both elimination rate and terminal competence. Thus, a role for protease appears quite likely at some level, but whether it acts *via* a postsynaptic mechanism remains to be determined.

CHAPTER 3

Differential Loss of Neuromuscular Connections According to Activity Level and Spinal Position of Neonatal Rabbit Soleus Motor Neurons

Abstract

We have tested whether the ability of synapses to compete for occupancy of endplates during neuromuscular synapse elimination is affected by differences in the spinal position or in the activity level of the parent motor neuron. To test the role of spinal position, the relative sizes of motor units for motor neurons from middle and extreme (rostral/caudal) positions in the rabbit soleus motor pool were determined at 3 postnatal ages: 4-5-days ("early" ages, when the soleus is heavily polyinnervated), 8-9-days ("intermediate"), and 11-15-days ("late," when the soleus has just reached the singly innervated state). Average motor unit sizes from extreme ventral roots were similar to those from middle ventral roots in early-aged soleus muscles but were significantly smaller for both intermediate and late muscles. Thus, motor neurons from extreme positions evidently compete less effectively for retention of synapses than those from middle positions.

To test the role of differential activity, inactive and active synapses were pitted directly against one another by implanting Silastic plugs laden with tetrodotoxin (TTX) into one of the spinal nerves containing a minority of the soleus motor axons. Differential activity was maintained during a period of extensive synapse loss, from the time of the implant at day 4 or 5 until the intermediate age (day 8-9). Motor unit twitch tensions were subsequently measured to determine the relative number of synapses retained by individual active and inactive motor neurons. The inactivated motor units were on average significantly larger than the corresponding group from normal and control-implanted animals. The increased size of inactivated motor units persisted in animals allowed to recover from the TTX block and examined after multiple innervation had disappeared. Hence, the effect of the TTX block cannot be attributed to a simple slowing of synapse elimination specifically among the inactive motor neurons. We conclude that complete presynaptic inactivity improves the

chances of survival relative to that for normal activity, during synapse elimination in the neonatal rabbit soleus muscle. This difference in competitive ability may contribute to the development of an important characteristic of adult muscles, the correlation between motor unit size and recruitment threshold.

Introduction

In the normal, newborn mammalian muscle, each muscle fiber is innervated at a single endplate by several different motor neurons. During the first weeks of postnatal development, the redundant synapses are lost, leaving each fiber innervated by exactly one motor neuron (Redfern, 1970; Brown et al., 1976).

Several observations indicate that the individual terminals at a polyinnervated endplate compete with one another for occupancy of the endplate. If the number of motor neurons innervating a muscle is reduced, the remaining neurons lose fewer synapses than is normal, indicating that motor neurons do not simply lose a predetermined number of synapses independent of interactions with other terminals (Brown et al., 1976; Thompson and Jansen, 1977; Betz et al., 1980; Fladby and Jansen, 1987). Competition is also a conservative hypothesis for explaining the observation that one-to-one connectivity develops without the appearance of denervated endplates. If competitive interactions do, in fact, occur, it is intriguing to consider what determines which synapse will survive at a given endplate. For example, are the connections from any particular motor neurons at a competitive advantage over the others? The experiments we describe here address several such possibilities, pertaining to the extent of arborization, rostral/caudal spinal position, and level of activity of motor neurons.

We assessed the role of spinal position by measuring twitch tension of motor units from different spinal roots as a function of developmental age during the period of synapse elimination in soleus muscles of normal rabbits. If the competitive ability of motor neurons were correlated with spinal position, then the relative sizes of motor units from different spinal roots should change with age. This possibility was first examined for the rat soleus muscle by Miyata and Yoshioka (1980), who suggested

that rostral motor neurons were at a disadvantage during synapse elimination, based on measurements of twitch tensions from whole ventral roots. Evidence against this hypothesis was subsequently obtained by measuring individual motor unit twitch tensions in soleus muscles of both rats (Thompson, 1983b; Gordon and Van Essen, 1983) and rabbits (Gordon and Van Essen, 1983). No significant rostral versus caudal difference in the extent of synapse loss was observed for either species; the difference observed by Miyata and Yoshioka was apparently due to a random difference in the proportion of soleus motor axons contributed by the ventral roots. However, small differences in synapse loss could have been obscured in these studies because the data were not analyzed separately for fast and slow units; it was not recognized until later (Gordon and Van Essen, 1985) that immature fast and slow motor units differ markedly in average size, at least in the rabbit soleus. We have therefore reevaluated the possibility of differential loss by spinal position, analyzing fast and slow motor units independently and by paying particular attention to the motor units at the rostral and caudal extremes of the soleus motor pool.

If a motor neuron's arbor size influences the extent of synapse loss, there should be a systematic change in the diversity of motor unit sizes as synapse elimination proceeds. For example, if neurons with smaller arbors were at an advantage, as has been suggested on theoretical grounds by Smalheiser and Crain (1984), initially small motor units would lose a smaller percentage of their synapses, while large units would lose proportionately more synapses. Accordingly, the diversity in motor unit sizes for the overall population would decrease. On the other hand, diversity would increase if large arbors were an advantage, a possibility suggested by the results of Gordon and Van Essen (1981). Once again, their investigations of this issue did not separate fast and slow motor unit types and therefore could not distinguish change in diversity within a group from a systematic change in the relative size of fast

and slow motor units. In the present study, we found that the relative size of the two populations indeed does change dramatically, but that there was no discernible change in the diversity within either population.

Activity has been strongly implicated in the regulation of the rate of synapse elimination. Blockage of nerve and muscle activity presynaptically, by botulinum toxin or tetrodotoxin (Brown et al., 1981; Thompson et al., 1979), or of muscle activity alone by α-bungarotoxin (Duxson, 1982; Chapter 2), dramatically slows or altogether halts synapse removal. Increased nerve and muscle activity, elicited by implanted stimulating electrodes, increases the rate of synapse elimination (O'Brien et al., 1978; Thompson, 1983a). However, these results do not address whether the level of activity of synapses affects their competitive ability, rather than simply governs the overall rate of the process. Will a difference in activity of synapses at the same endplate influence which synapse will remain? If the neuromuscular junction were regulated by Hebb-like rules of competition (Hebb, 1949), more active inputs should outcompete less active inputs, as has been suggested by Ridge and Betz (1984) and Ribchester and Taxt (1983). Contrary to this prediction, our results indicate that inactivity actually increases the survival probability of a synapse when it is in direct competition with active synapses.

Methods

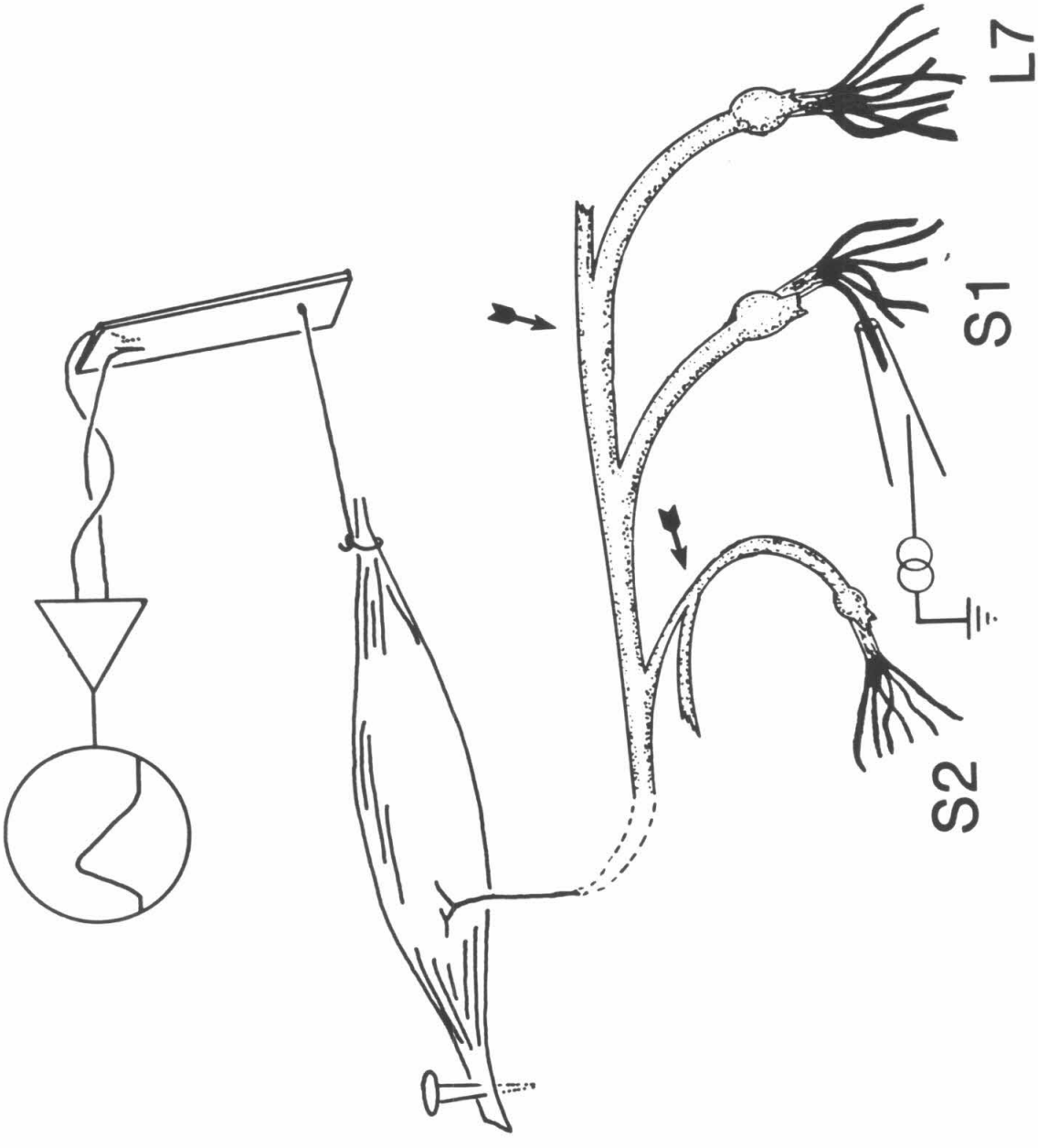
Twitch Tension Recording. After initial sedation with Metofane, animals were heavily anesthetized with ether, and the legs and lower spinal column were removed and placed in chilled Ringer's solution. The soleus muscle was dissected free along with its contributing innervation up to and including the ventral roots L7, S1, and S2 (Fig. 1).

Twitch tensions resulting from stimulation of each of the whole ventral roots contributing to the soleus muscle were determined. Roots were then split into 10-30 filaments, and twitch tensions and rise times of individual motor units were determined by graded stimulation of individual filaments of ventral roots containing 4 or fewer soleus motor axons. Twitch responses were measured with a tension transducer attached to the muscle's distal tendon by a length of 6-0 silk thread (Fig. 1). The electrical signals from the transducer were amplified 10-1000X and sampled with an IBM PC-XT computer equipped with a Tecmar analog-to-digital conversion board. Programs for both on-line and off-line analysis were available. When a filament contained more than one soleus motor axon, the summed responses were subtracted to reveal the twitch responses of the individual motor units. Filaments containing more than 4 soleus motor axons were split further in order to minimize error that might occur due to overlap of motor units and to non-linear summation of twitch responses (Brown and Matthews, 1960). We also measured the twitch tensions elicited by direct, supramaximal stimulation of the whole muscle. The experiment was discontinued if the maximal response dropped below 80% of its original value, and maximal responses were measured frequently enough so that the value seldom varied by more than 5% between samples.

In general, every motor unit was measured in ventral roots containing fewer than

Figure 1. Diagram of the preparation for *in vitro* measurement of rabbit soleus muscle twitch responses. The preparation includes the rabbit soleus muscle pinned at its proximal tendon to the bottom of a wax-lined dish and its contributing innervation as far proximal as the ventral roots (L7, S1, and S2), tension transducer attached to the distal tendon via 6-0 silk, and suction electrode for stimulation of teased filaments of ventral roots. Analog signals from the tension transducer were amplified, observed on a CRO (not shown), converted to digital signals and analyzed both on- and off-line by computer (not shown). Arrows indicate location in L7 or S2 spinal nerve where a TTX-laden or control plug was implanted in some animals.

FIGURE 1



20 soleus motor axons, but it was usually necessary to terminate the experiment before all motor units from the S1 ventral root could be assayed. Typically, a total of 30-50 motor units were assayed per muscle. Measurements from the various ventral roots were interspersed in order to avoid artifactual size differences of motor units that might result from systematic drifts in recording conditions or state of the preparation over time. Complete interspersal was difficult to achieve, though, especially for roots having only a few motor units. To address this issue further, we occasionally assayed an identified motor unit again at the conclusion of an experiment (1-2.5 hours later). In 7 of 9 instances of this type, the change in measured size was less than 12% (similar to the variation attributable to noise with repeated successive measurements); in the other 2, the decline was somewhat larger. Also, when the mean size of the first 5 fast or slow motor units measured from the S1 root of each animal was compared to the mean of the last 5, there was no systematic tendency for measured tensions to increase or decrease. Overall, the last 5 units measured were smaller than the first by only 4% for fast and 12% for slow; neither difference is statistically significant ($p > 0.2$ for each comparison). Even if these small differences were meaningful, a modest decline in mean sizes over the course of an experiment would work against the observed differences in size between motor units from different roots (see Results). For example, we found that in normal animals the motor units from the minor contributing roots were relatively small, even though they tended to be assayed early during the experiments. For TTX-implanted animals, the motor units from the implanted roots were measured relatively late in the sequence (owing to the need to wait for TTX washout at the intermediate age and explicitly to avoid the possibility of bias at the late age); nonetheless, their mean sizes were relatively large. Additional aspects of tension recordings are described in detail elsewhere (Gordon and Van Essen, 1983; 1985).

Separation of motor unit twitch types. The distinction between fast and slow motor units was based mainly on the bimodal distribution of twitch rise-times characteristic of rabbit soleus muscles at all postnatal ages (Fig. 2; see also Gordon and Van Essen, 1985). In some muscles, the best split point was not obvious (even though the distribution of rise times was bimodal), resulting in uncertainty about the assignment of several ($\leq$ 5) motor units. To assign these borderline motor units more accurately, we took advantage of the approximately twofold difference in mean, motor unit twitch-tension between fast and slow groups (Gordon and Van Essen, 1985; see also Fig. 2); the initially ambiguous units were assigned to the group whose range of sizes best matched the unit in question. Even if this led to inappropriate assignment of a few motor units in our overall sample, none of our major conclusions would be affected (see Discussion).

Grouping of animals. Rabbits were categorized according to their age at the time of *in vitro* physiology (early, intermediate or late), experimental treatment (normal, control-implanted, or TTX-implanted), and the distribution of soleus motor axons amongst the ventral roots (Table 1).

The ages examined were 4-5 days postnatal for the early group and 8-9 days for the intermediate group. The late group included 11-15 day normal animals but only 14 and 15 day animals for control and TTX-implanted groups.

In all muscles, the innervation to the soleus arose from two or three spinal roots. The S1 root invariably provided the majority of the inputs, as judged by comparing the overall tension elicited from stimulation of different roots and/or by counting the number of motor units isolated from each root. In about half of the cases (17 of 33 normal muscles), the additional inputs arose from both L7 and S2 roots; in the other cases, the additional inputs usually arose from L7 alone (12 cases) but

Figure 2. Plot of twitch rise-times against sizes of motor units expressed as percentage tension from a normal, 8-day-old animal. Mean percentage tension motor unit sizes were 6.4± 0.4% and 2.5± 0.1% for fast and slow units, respectively. Mean rise-times were 218± 3 msecs and 368± 8 msecs for fast and slow units, respectively. The fastest rise time for a slow unit was 291 msecs, and the slowest rise time for a fast unit was 257 msecs. Note that the rise times of these motor units are slower than typical *in vivo* values because of the lower temperature at which our *in vitro* measurements are made.

FIGURE 2

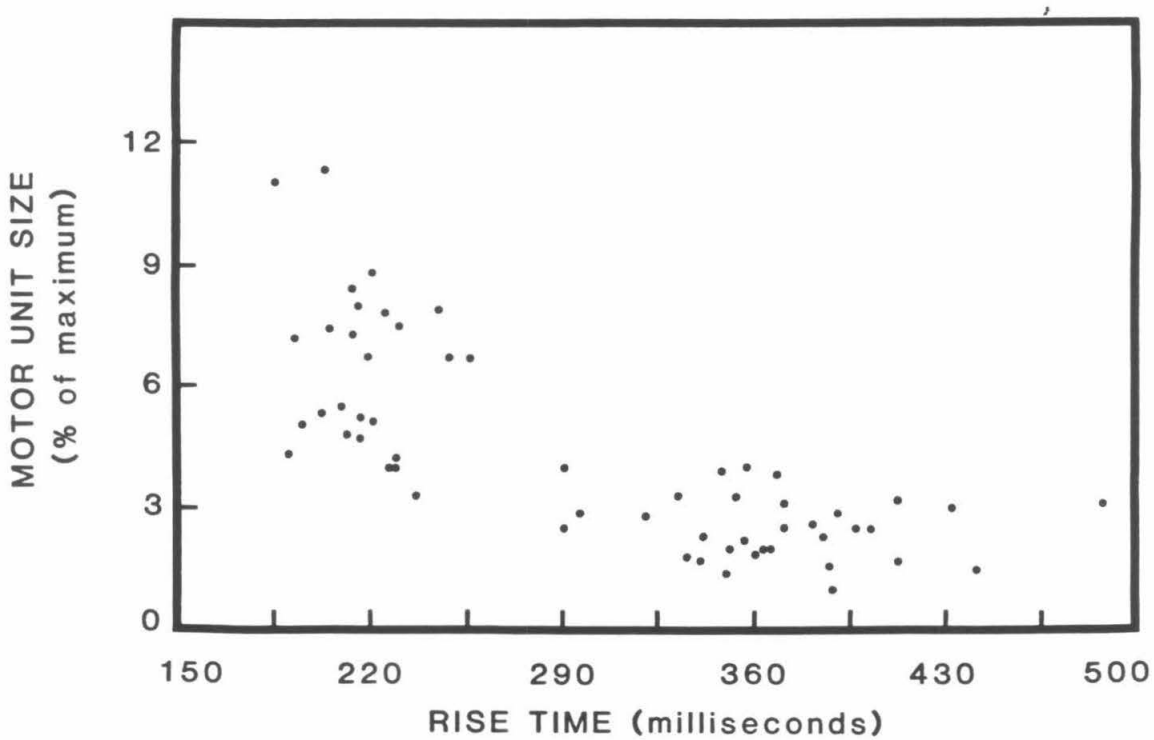

MOTOR UNIT SIZE
(% of maximum)
12
9
6
3
0
150
220
290
360
430
500
RISE TIME (milliseconds)

Table 1. Number of animals by experimental group – normal, control, and TTX-implanted at early, intermediate, and late ages – and by ventral roots contributing to the soleus innervation. For normal animals, the first number in each group represents the number of animals in that group whose soleus innervation was contributed by the S1 and L7 ventral roots; the second number corresponds to the number of animals with soleus contributions from S1 and S2; and the third number is the number of animals with soleus contributions from all 3 ventral roots. For TTX- and control-implanted animals, the first number for each group corresponds to the number of animals whose L7 spinal nerve was implanted and the second, the number of animals with an S2 implant. Only animals for which there was a soleus contribution from the implanted nerve are included. Some animals were not analyzed and are not included in the above table because of one of the following: Fewer than 5 middle motor units of each twitch type were assayed before the *in vitro* preparation degraded, equipment malfunction made motor unit size estimates inaccurate, or the extreme root had too many motor units to be considered a minor root, so motor unit sizes were not assayed. Three animals for which the extreme root was not a minor root are included in parentheses for S2 TTX-implanted animals at the intermediate age; these animals were analyzed and are considered separately as TTX-implanted, major-block animals in the results section.

TABLE 1

	Normal			Control		TTX-implanted	
	L7	S2	L7&S2	L7 implant	S2 implant	L7 implant	S2 implant
Early (4-5 days)	2	0	5	--	--	--	--
Intermediate (8-9 days)	0	3	3	6	4	5	4 (3)
Late (11- or 14-15 days)	4	1	4	9	0	11	0

sometimes were from S2 alone (4 cases). For simplicity, we will refer to L7 and S2 as "extreme roots" and S1 as the "middle root", even for muscles supplied by only two roots.

In the experiments aimed at studying the effects of differential activity, our experimental paradigm required that active and inactive synapses compete directly against one another on active muscle fibers. For this condition to be met, the contribution from the implanted nerve had to be small, so that at the time of the initial TTX implant, each muscle fiber with an inactive input would also receive one or more active inputs. We therefore analyzed the innervation of normal 4 to 5-day rabbits to determine how many soleus motor axons an extreme root could contain while still having all of its target fibers co-innervated by the remaining roots. We found that the overlap in innervation at this early age was complete as long as the extreme root contained 18 or fewer soleus motor axons (see results). This was the case in the great majority of animals examined; extreme roots meeting this condition were termed "minor roots". In about 20% of the animals (15/89), one of the extreme roots was estimated to contain more than 18 soleus motor axons and was termed a "major root". The 3 cases in which a TTX block was maintained for at least 4 days in a major root were analyzed separately from the rest of the data. In normal and control animals, cases with soleus innervation from major roots were discarded without further analysis, because only the minor root cases formed an appropriate basis for comparison with the main group of TTX-implanted animals.

Analysis of twitch tension data. In this study we were mainly interested in comparing the average size of motor units originating from different spinal roots (e.g., extreme versus middle roots; active versus inactive roots). We based our analysis on two different expressions for motor unit twitch-tension. The first is the "percentage tension", which involves scaling to the maximal tension elicited by

direct muscle stimulation:

$$t_i = 100\theta_i/\Theta_{max}, \tag{1}$$

where θ_i is the twitch tension in grams of the i^{th} motor unit, Θ_{max} is the maximal direct tension in grams, and t_i is the percentage tension.

Scaling to a percentage value was intended to compensate for the large variability in maximal tension produced by different muscles, even for muscles from animals of similar age. However, even after this initial compensation, large (twofold or more) differences persist in the average percentage tension of both fast and slow motor units from different muscles (see Results). Hence, for much of our analysis, we used an additional normalization step in which the motor unit sizes were scaled to the median value for the corresponding contractile type from the middle root of the same animal:

$$\phi_i = t_i/T^{s,f}_{mm}, \tag{2}$$

where ϕ_i is the "normalized tension" and T^s_{mm} and T^f_{mm} are the median percentage tension values for the fast and slow motor units from the middle root of the same animal. Scaling was done to medians rather than to means because subsequent statistical analyses were carried out using non-parametric tests. In order to assure that the median values estimated for each animal gave an adequate estimation of the true median value, only animals from which at least 5 middle root motor units of each twitch type were measured were included in our analysis.

The normalized tension emphasizes a motor unit's size relative to the typical middle root motor unit in the animal from which it was measured, and it provides a satisfactory basis for pooling results from different animals. For statistical analysis of normalized tension values, all the appropriate values from all the animals in a group were pooled together, and comparisons of those populations were made with

the Mann-Whitney U-test or the Kolmogrov-Smirnoff Test. The overall, mean, normalized tension value for each group was thus calculated by

$$\Phi = (\sum_i \sum_m \phi_{im})/n_{tot}, \tag{3}$$

where ϕ_{im} is the value of ϕ for the i^{th} unit from the m^{th} muscle, n_{tot} is the total number of units, and Φ is the mean, normalized tension for the experimental group.

When it was necessary to compare absolute measures of motor unit size without normalization to the median (i.e., using percentage tension values), it was not appropriate to pool the values from different animals and treat them as independent samples – the large variation in mean sizes between animals made each value dependent on the animal from which it was measured. We therefore calculated the mean, percentage tension values from individual animals:

$$T_m = (\sum_i t_i)/n, \tag{4}$$

where n is the number of units and T_m is the mean percentage tension for the m^{th} muscle. These mean, percentage tension values were pooled for the various experimental groups, and their distributions were compared using the Mann-Whitney U-test. The overall, mean, percentage tension value for each group was calculated by

$$T_{tot} = (\sum_m T_m)/n, \tag{5}$$

where n was the number of muscles in the experimental group and T_{tot} was the overall, mean, percentage tension.

Variables in Equations 1-5 represented by lower-case symbols indicate that they represent a size estimate based on the twitch response of a single motor unit, while representation by upper-case symbols indicates values calculated from a population of motor units. These populations do not always include all of the motor units

from a given muscle or group of muscles; motor units from middle or extreme roots and for fast and slow populations are considered separately for most of the analyses. For example, in Equation 2, T^s_{mm} refers to the median percentage tension for the population of slow motor units from the middle root of a particular animal. In Equations 4 and 5, the upper-case "T" represents a mean value rather than a median, either of the percentage tension values of individual motor units (t_i) from a single muscle or of the mean, percentage tension values (T_m) from a group of muscles.

TTX and control plug manufacture. TTX-laden plugs were manufactured by the method developed by Gordon (1984). A one-to-one mixture of α-cellulose and collagen fibrils suspended in water was applied to the tip of a 2-3mm length of 40-gauge platinum wire. After about 15 min drying, 0.20 to 0.23 μl of 10 mM TTX was applied to the α-cellulose/collagen and allowed to dry 1 h. Plugs were then dipped into Silastic thinned with xylene so that a smooth, thin coat around the TTX-impregnated matrix acted as a time-release diffusion barrier. Before use, plugs were cured for 3 days at room temperature and at least 4 additional days at 5°C. Potency was consistent among plugs made at the same time and under the same conditions. However, despite careful measurement of the amount of TTX applied, the potency of different batches of plugs was quite variable. With overly potent plugs, the animals failed to recover from anesthesia; with under-potent plugs there was no muscle paresis in the behavioral assay described below. If a batch of plugs proved too potent when first implanted, the remaining plugs were stored at 5°C for several weeks and were implanted when the Silastic had cured further and when perhaps some of the TTX had degraded. Control plugs were made identically except that no TTX was applied.

Plug implantation. A control or TTX-laden plug was surgically implanted into the

left L7 or S2 spinal nerve of 4 or 5-day-old rabbits under ketamine anesthesia. For S2 implantation, the spinal nerve was exposed by incision through the skin and musculature alongside the dorsal spinal cord just above the nerve's exit from the vertebra, and a plug was implanted just proximal to the junction at the sciatic plexus. L7 implantations were achieved by a ventral approach just lateral to the lower abdominal cavity; a plug was implanted into the L7 spinal nerve between its junctions with the L6 and S1 spinal nerves. Plugs were inserted into the appropriate nerves through a small hole in the epineurium made with a fine tungsten needle. After allowing at least 2 hours for recovery, rabbits were returned to a hutch with their littermates to be cared for by their mothers.

Assays for TTX induced activity block. The effectiveness of nerve impulse blockade in TTX-implanted nerves was assessed both *in vitro* and *in vivo*. The *in vitro* assay was the more direct, as it tested the ability of a specific nerve to propogate action potentials past the implant site. All TTX and control-implanted animals were assayed for conduction block of their implanted nerve *in vitro* during and/or after dissection of the soleus muscle and its innervation. The implanted nerve was stimulated both proximal and distal to the implant with a roving bipolar stimulating electrode (5-15 volts, 0.1-0.2 msec). The nerve was considered blocked if the distal stimulation induced visible muscle contraction (not necessarily limited to the soleus muscle), but proximal stimulation did not. For animals that proved blocked based on this assay, the TTX plug was removed, and motor unit twitch responses were measured after conduction was restored.

Since our *in vitro* assessments were made at about 14-19°C, they do not necessarily indicate that conduction was fully blocked at higher temperatures *in vivo*. We therefore used an *in vivo* assay as well. Animals were inspected for left-(implanted) versus right-side asymmetry in foot position at rest and in resistance

to extension. *In vivo* assays of S2-implanted animals proved unreliable because of the small number of motor axons in that nerve and its primary innervation of tail musculature. However, *in vivo* assays of L7 implanted animals proved very reliable. L7-blocked animals were characterized by a moderate-to-strong resting extension of the left foot relative to the right when the animal was held on its back, and by little or no reflexive counter-flexion in response to passive extension of the foot. The reliability of this assay is evidenced by comparison of *in vivo* and *in vitro* assessments of the state of blockade made in succession on the same day. Fourteen animals were judged to be blocked by the *in vivo* assay 4 days after the L7 nerve (or sometimes S1 or L6 inadvertently) had been implanted with a TTX plug. In each of these cases, the implanted nerve was also blocked based on the *in vitro* assay. An additional 2 animals were blocked *in vitro*, but not *in vivo* on the fourth day: one, a block of L7, had been clearly blocked *in vivo* for 3 days; the other was a block of S1, which showed only weak evidence of *in vivo* block for 1-2 days. Dozens of other TTX-implanted animals were blocked based on the *in vivo* assay for 1-3 days but were not blocked based on either the *in vivo* or the *in vitro* assay on the fourth day. TTX-implanted animals that were not blocked based on the *in vitro* assay at the time of dissection were discarded without further analysis.

No control-implanted animals showed evidence of conduction block in the implanted nerve based on either of the assays. No animal showed evidence of TTX block beyond 5 days after implantation. Therefore, for the long-term TTX series, animals were dissected at days 14-15 (10-11 days after implantation), only L7 was implanted, and only animals blocked for 4-5 days by the *in vivo* assay were dissected and analyzed.

Silver staining of nerve terminals. Muscles in the late age control and TTX-implanted groups were stained by a modification of the silver-cholinesterase method

described by Hopkins et al. (1985). Following fixation and storage in buffer solution as they describe, muscles were cut longitudinally into 40μm sections on a freezing microtome. Sections were mounted on gelatin-subbed slides. Mounted sections were placed in cold Karnovsky AChE stain (Karnovsky and Roots, 1964) with no substrate for 6-7 minutes, then washed in water for 5 minutes. Sections were then stained for esterase to mark endplates for 15-20 minutes at 37°C, as described by Pestronk and Drachman (1978). This stain produces a transparent blue reaction product that allows visualization of nerve terminals within the esterase-stained region. Sections were subsequently processed as described by Hopkins et al. (1985).

One hundred endplates from each muscle were observed at 400X magnification, and the number of axons contributing to the innervation of the endplate was determined. Slides were coded so that the observer did not know whether a given muscle was from the TTX- or control-implanted group. Endplate-rich regions of muscle sections were examined at 400X. Every endplate, as defined by esterase staining in the resulting field was examined, but only complete endplates not at the edges of the section plane (determined by focus) were scored. When all endplates in the field had been observed, the stage was moved to bring more endplates into view, and every additional endplate was characterized. Typically 30 to 60 endplates were observed per section.

Results

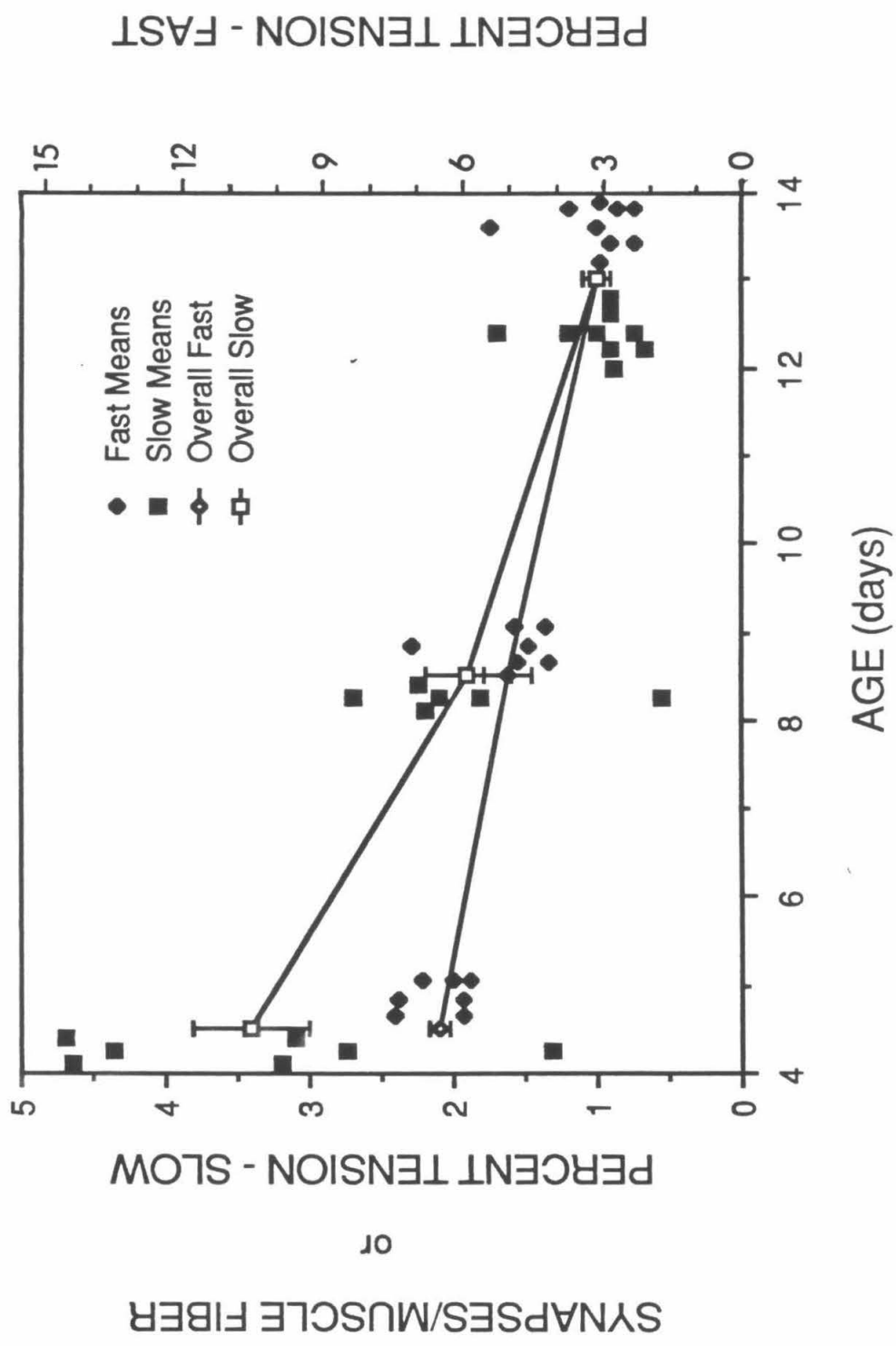
Normal Animals

Changes in polyinnervation by fiber type. We will begin by analyzing overall changes in fast and slow motor units among normal animals without regard for spinal position. Figure 3 shows the mean percentage tension of fast and slow motor units from S1, the middle root (T_m^s and T_m^f) from each normal animal (filled diamonds and filled squares for fast and slow means, respectively), along with overall means ($\pm$ S.E.M.) for each age group (open diamonds and open squares for fast and slow, respectively). The fast and slow means are plotted on different scales, which were adjusted to make the overall means coincide at the late age. At the early age, the overall mean percentage tension values were $6.5 \pm 0.2\%$ for fast units versus $3.4 \pm 0.4\%$ for slow. These values decreased to $5.0 \pm 0.5\%$ versus $1.9 \pm 0.3\%$ at the intermediate age and to $3.1 \pm 0.3\%$ versus $1.0 \pm 0.1\%$ at the late age (see also Table 2, column B). Thus, mean percentage tension was greater for fast than for slow motor units for every animal at every age (although in a few cases the mean slow value from one animal was larger than the mean fast size from a different animal of the same age). This confirms the observations made by Gordon (1984). This difference could result from a greater number of muscle fibers innervated by each neuron for the fast population or from generation of more tension per muscle fiber for the fast fibers.

A second independent measure of motor unit size suggests that the tension generated per fiber is similar for fast and slow fibers and that fast neurons innervate about twice as many fibers as slow. Since there are about twice as many Type II (presumed fast-contracting) than Type I (presumed slow-contracting) fibers in the soleus at the ages in question, but roughly equal numbers of fast and slow motor

Figure 3. Mean motor unit sizes expressed as percentage tension from middle roots of individual early, intermediate and late-age animals, and degree of polyinnervation expressed as synapses per muscle fiber based on those values. Filled diamonds represent mean values for fast motor units and filled squares for slow units from individual animals. Open symbols represent overall mean values. The same scale for synapses per muscle fiber is used for both fast and slow values, but the percentage tension values are scaled differently for the two populations, so that both overall mean values correspond to unity on the polyinnervation scale at the late age. Note that for the slow population, the percentage tension scale is the same as the synapses per muscle fiber since the overall slow percentage tension mean at the late age was 1.0. Each filled diamond represents the mean, percentage tension size of fast motor units from a single animal. The mean values for early-age animals range from 5.9% to 7.5% with an overall mean (open diamond) of 6.5±0.2%. The range for intermediate-age animals was 4.1% to 7.1% with a mean of 5.0±0.5% and for late-age animals was 2.3% to 5.4% with a mean of 3.1±0.3%. The overall mean values correspond to values of 2.1, 1.6, and 1.0 synapses per muscle fiber on the polyinnervation scale for early, intermediate, and late ages, respectively. Filled squares represent mean sizes of slow motor units from individual animals. For early-age animals mean values range from 1.3% to 4.7% with an overall mean (open square) of 3.4±0.4%. Ranges were 0.54% to 2.7% for intermediate-age animals with a mean of 1.9±0.3%, and 0.66% to 1.7% for late-age animals with a mean of 1.0±0.1%. The overall mean values correspond to values of 3.4, 1.9, and 1.0 synapses per muscle fiber at early, intermediate, and late ages, respectively.

FIGURE 3


PERCENT TENSION - FAST
15
12
9
6
3
0
Fast Means
Slow Means
Overall Fast
Overall Slow
5
4
3
2
1
0
PERCENT TENSION - SLOW
4
6
8
10
12
14
AGE (days)
or
SYNAPSES/MUSCLE FIBER

neurons, it follows that each fast neuron should innervate about twice as many muscle fibers as slow neurons (Gordon, 1984). This difference is comparable to the differences in overall, mean, twitch tensions we observe. Assuming that there were no major differences in the degree of non-specific innervation (e.g., fast muscle fibers by slow motor neurons and slow fibers by fast neurons), it follows that the tension generated per muscle fiber is similar for fast and slow fibers.

The greater tension produced by fast motor units is particularly noteworthy given that Type II muscle fibers are much smaller than type I fibers (Gordon, 1984). If, as concluded above, the average tension generated per fiber is similar for fast and slow fibers, there must be a large disparity in specific tension (per unit area) generated by fast versus slow fibers as well as the twofold difference in motor unit size.

Since nearly all muscle fibers are singly innervated at the late age (Bixby and Van Essen, 1979), the ratio of percentage tensions between early and late ages provides an estimate of the average degree of polyinnervation of each fiber type. This ratio is indicated by the scale on the left of Figure 3. Interestingly, the estimated rate of synapse loss between early and late ages is about twofold greater for the slow than the fast fibers (.28 versus .13 synapses/fiber/day). Also, the rate of elimination appears to be similar during the different time periods we tested for the fast fibers (.12 syn./fiber/day between early and intermediate ages versus .14 between intermediate and late) but much faster during the earlier than during the later interval for the slow fibers (.38 syn./fiber/day versus .20). It is important to note, however, that these estimates of the rate of synapse elimination are predicated on the unproven assumption that the relative tension generated by individual fast and slow muscle fibers does not change during this maturational period.

It is apparent from Figure 3 that there was considerable variability from one muscle to the next in the average percentage tension for each contractile type and each age group. The ratios of the largest and smallest average percentage tension for a given contractile type and age group ranged from a factor of nearly two- to more than fivefold. Some of this variability must arise from sampling errors, given that we assayed only a fraction of the whole motor unit pool and that there was considerable dispersion of motor unit tensions within each muscle (cf. Fig. 2 and below). An analysis of variance, however, indicated that the differences between muscles were statistically significant and were not entirely attributable to incomplete sampling.

Knowing the specific sources of this variability is not critical to our analysis, but it is worthwhile to mention some of the more obvious candidates for intrinsic biological variability and for systematic measurement errors. Possible sources of biological variability include individual differences in the total numbers of motor axons or muscle fibers, in the relative numbers of fast and slow motor neurons and of fast and slow fibers, and in the degree of polyinnervation. The most likely source of measurement error stems from underrepresentation of maximal tension that may occur due to non-isometric recording conditions (Brown and Matthews, 1960). This would result in an overestimate of the percentage of soleus muscle fibers innervated by individual motor neurons as estimated by the percentage tension. We estimated the degree of this error and its variability by multiplying the average percentage tension from middle roots of late-age muscles by the number of soleus motor neurons, estimated to be about 70 by Bixby and Van Essen (1979). This value averaged 141±35% (mean ± S.D.) for the 18 late-aged normal and control muscles, whereas it should be precisely 100% if there were no residual polyinnervation and if there were perfect isometric recording conditions.

The key point for our subsequent analysis is that the variability within animals

and that between animals comprise two distinct sources of statistical fluctuations that need to be handled separately. We achieved this separation in two ways. In the first method, we pooled the data on motor unit tensions only after scaling to the median value for the middle root from the same animal (see Methods, Equations 2 and 3). This normalization procedure largely removes the contribution of individual variability, whether it be from measurement errors or of biological origin. It thereby allows for sensitive comparisons, since motor units from different muscles (but from same root and contractile type) can be treated as independent samples from a single distribution. In the second method, we calculated the mean, percentage tension values for each root and contractile type from each muscle (see Methods, Equation 4); if a mean, percentage tension value for a group of motor units from different muscles was desired, it was calculated by pooling the means from the individual muscles (Equation 5). While percentage tension is a more direct measure than normalized tension, it is inherently less sensitive to small (but genuine) differences in the relative sizes of motor units from the same animal.

Changes in extreme relative to middle motor unit sizes. In comparing motor units from different spinal roots, we found that the normalized motor unit tensions from extreme roots (L7 & S2) were significantly smaller on average than those from the middle root (S1) at both intermediate and late ages. This difference was not present in the early age group. These findings are illustrated in Figure 4, which shows bar histograms of mean, normalized motor unit tensions, with error bars indicating the standard error of the mean. In addition, Table 2 tabulates mean values with standard errors, expressed as normalized tension (relative to the middle root median; column A) and as percentage tension (relative to maximal twitch tension; column B).

For fast motor units (Fig. 4a), the normalized tensions from extreme roots

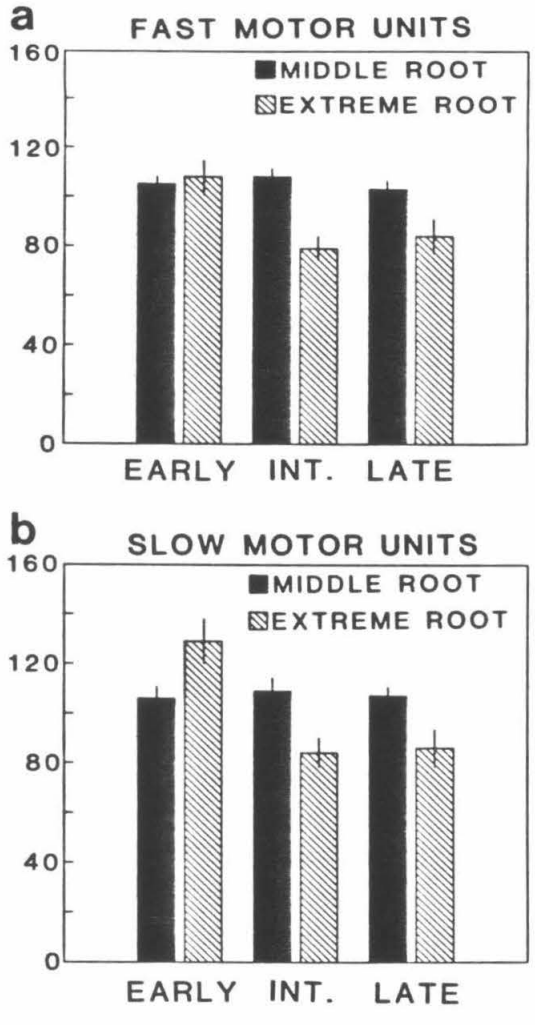
Table 2. Normalized values represent mean ± S.E.M. of the pooled motor unit sizes, expressed as percentage of middle root median, from all animals in the group. Numbers in parentheses are number of motor units. The normalized values from middle roots exceed 100% because distributions of motor unit sizes are skewed such that the mean is greater than the median (see Figs. 5-7). Percentage tension values represent mean ± S.E.M. of the mean values from each animal in the group (values shown in Fig. 3). Numbers in parentheses represent the number of animals (means) in each group. The number of animals for rostral, caudal, and extreme root groups is sometimes smaller than for middle root groups because not every animal has soleus contribution from both rostral and caudal roots and some extreme roots contributed unit(s) of one twitch type but not the other. [a]Equal to same age, normal, middle root; $p > 0.2$. [b]Larger than same age, normal, middle root; $p < 0.05$. [c]Smaller than same age, normal, middle root; $p < 0.001$. [d]Smaller than same age, normal, middle root; $p < 0.05$. [e]Equal to same age, normal, rostral root; $p > 0.2$. [f]Equal to same age, normal, rostral root; $p > 0.1$. [g]Smaller than early age, normal, extreme root; $p < 0.001$. [h]Smaller than early age, normal, extreme root; $p < 0.005$. [i]Equal to intermediate age, normal, extreme root; $p > 0.2$. [j]Equal to same age, normal, extreme root; $p > 0.2$. [k]Larger than same age, control-implanted, extreme root; $p < 0.001$. [l]Larger than same age, control-implanted, extreme root; $p < 0.02$. [m]Equal to intermediate age, TTX-implanted, extreme root; $p > 0.2$. [n]Equal to same age, control-implanted, middle root; $p > 0.2$. [o]Smaller than same age, control-implanted, middle root; $p < 0.05$. All p values determined from Mann-Whitney U-test.

TABLE 2

		A Normalized Tension		B Percentage Tension	
		Fast	Slow	Fast	Slow
	4-5d Normal Animals:				
1	**Middle Roots**	$105 \pm 3(138)$	$106 \pm 5(88)$	$6.5 \pm 0.2(7)$	$3.4 \pm 0.4(7)$
2	**Rostral Roots**	$109 \pm 9(22)$	$139 \pm 10(20)$	$7.2 \pm 1.3(5)$	$4.1 \pm 0.4(7)$
3	**Caudal Roots**	$107 \pm 12(12)^{e}$	$112 \pm 19(11)^{e}$	$7.1 \pm 1.3(3)$	$3.5 \pm 0.7(5)$
4	**Extreme Roots**	$108 \pm 7(34)^{a}$	$129 \pm 9(31)^{b}$	$6.7 \pm 0.9(7)$	$3.8 \pm 0.4(7)$
	8-9d Normal Animals:				
5	**Middle Roots**	$108 \pm 4(101)$	$109 \pm 5(115)$	$5.0 \pm 0.5(6)$	$1.9 \pm 0.3(6)$
6	**Rostral Roots**	$93 \pm 10(4)$	$84 \pm 9(19)$	$3.7 \pm 0.1(2)$	$1.4 \pm 0.3(3)$
7	**Caudal Roots**	$77 \pm 5(23)^{f}$	$83 \pm 6(16)^{e}$	$3.8 \pm 0.4(5)$	$1.7 \pm 0.2(4)$
8	**Extreme Roots**	$79 \pm 5(27)^{c,g}$	$84 \pm 6(35)^{c,g}$	$3.9 \pm 0.3(6)$	$1.6 \pm 0.2(6)$
	8-9d Control Animals:				
9	**Middle Roots**	$100 \pm 3(201)$	$101 \pm 3(165)$	$5.7 \pm 0.7(10)^{a}$	$2.3 \pm 0.3(10)^{a}$
10	**Extreme Roots**	$73 \pm 5(32)^{j}$	$91 \pm 7(29)^{j}$	$3.9 \pm 0.5(10)$	$1.8 \pm 0.2(10)$
	8-9d TTX Animals:				
11	**Middle Roots**	$102 \pm 3(165)$	$105 \pm 5(105)$	$4.6 \pm 0.6(9)^{a,n}$	$1.6 \pm 0.2(9)^{a,o}$
12	**Extreme Roots**	$106 \pm 7(28)^{k}$	$137 \pm 11(26)^{k}$	$4.9 \pm 0.8(9)$	$2.1 \pm 0.3(9)$
	11-15d Normal Animals:				
13	**Middle Roots**	$103 \pm 4(167)$	$107 \pm 4(152)$	$3.1 \pm 0.3(9)$	$1.0 \pm 0.1(9)$
14	**Rostral Roots**	$89 \pm 9(20)$	$90 \pm 9(18)$	$2.6 \pm 0.7(5)$	$1.0 \pm 0.2(4)$
15	**Caudal Roots**	$70 \pm 11(8)^{e}$	$77 \pm 12(10)^{e}$	$2.3 \pm 0.2(6)$	$0.80 \pm 0.1(8)$
16	**Extreme Roots**	$84 \pm 7(28)^{d,h,i}$	$86 \pm 7(28)^{d,g,i}$	$2.5 \pm 0.4(9)$	$0.87 \pm 0.1(9)$
	14-15d Control Animals:				
17	**Middle Roots**	$105 \pm 3(132)$	$108 \pm 3(179)$	$3.2 \pm 0.2(9)^{a}$	$0.87 \pm 0.1(9)^{a}$
18	**Extreme Roots**	$80 \pm 5(26)^{j}$	$77 \pm 5(34)^{j}$	$2.7 \pm 0.1(8)$	$0.64 \pm 0.1(9)$
	14-15d TTX Animals:				
19	**Middle Roots**	$106 \pm 3(190)$	$110 \pm 4(155)$	$3.0 \pm 0.2(11)^{a,n}$	$0.73 \pm 0.1(11)^{d,n}$
20	**Extreme Roots**	$110 \pm 11(21)^{l,m}$	$168 \pm 21(27)^{k,m}$	$2.7 \pm 0.3(8)$	$1.1 \pm 0.1(9)$

Figure 4. Comparisons of normalized fast motor unit sizes (a) and slow motor unit sizes (b) from middle and extreme roots of early, intermediate, and late-age animals. Bar heights represent mean± S.E.M. normalized motor unit sizes (percentage of middle root median). **a.** Fast motor units from middle and extreme roots are equal in size at early ages. Extreme root motor units are significantly smaller than middle root motor units at both intermediate and late ages. Extreme root motor units at both intermediate and late ages are significantly smaller than extreme root motor units at early ages. Motor units from extreme roots at intermediate and late ages are equal in size. **b.** Slow motor units from middle roots are significantly smaller than those from extreme roots at early ages. Extreme root motor units are significantly smaller than middle root motor units at both intermediate and late ages. Extreme root motor units at both inmtermediate and late ages are significantly smaller than extreme root motor units at early ages. Motor units from extreme roots at intermediate and late ages are equal in size. If separate statistical comparisons of normalized mean sizes for rostral and caudal motor units against middle motor units were made, the only significant difference ($p < 0.05$, Mann-Whitney U-test) at the early age was for the slow, rostral units ($139 \pm 10\%$, Table 2, 2A) which were larger on average than the slow, middle units ($106 \pm 5\%$, Table 2, 1A). At intermediate and late ages, each of the 4 differences (fast and slow at each age) for caudal versus middle motor units was statistically significant with caudal means smaller than middle. However, for rostral versus middle motor units there was a statistically significant difference for only 1 of the 4 comparisons – slow motor units at the intermediate age. The differences between rostral and middle motor units were statistically significant for both the fast and slow populations, if the values from the intermediate and late age groups were pooled. This was also the case for both intermediate and late ages, if fast and slow values were pooled. See Table 1 for mean values and significance of comparisons.

FIGURE 4



MOTOR UNIT TWITCH STRENGTH
AS % OF MIDDLE ROOT MEDIAN
MEAN PLUS/MINUS S.E.M.

(hatched bars) and middle roots (solid bars) were not significantly different on average at early ages (108% versus 105%, $p > 0.2$, Mann-Whitney U-test). At the intermediate age, however, fast motor units from extreme roots were on average 27% smaller than those from middle roots (79% versus 108%, $p < 0.001$). The difference was not as large (18%) for 11-15 day old animals (84% for extreme versus 103% for middle roots), but it was still statistically significant ($p < 0.05$).

The results for the slow population (Fig. 4b) were similar to those from the fast population, except that at early ages, extreme root motor units were actually larger than those from middle roots by a significant amount (129% versus 106%, $p < 0.05$). Again, comparing hatched to non-hatched bars from the same age, slow motor units from extreme roots were on average 23% smaller than those from middle roots at intermediate ages (84% versus 109%, $p < 0.001$) and 20% smaller at late ages (86% for extreme versus 107% for middle, $p < 0.05$).

By comparing the hatched bars at different ages, it is apparent that the relative size of extreme motor units declines between early and intermediate ages and then remains stable between the intermediate and late ages. This initial decline is somewhat larger for the slow population (from 129% to 84%) than for the fast population (from 108% to 79%).

These results suggest that synapse elimination initially proceeds faster for motor neurons from extreme roots compared to those from the middle root. But how much of a difference is this in relation to the overall rate of synapse elimination in either root? To address this issue, we used percentage tension values (Table 2, column B) to estimate the overall decline in motor unit sizes for middle and extreme roots. For the fast population, the decline in motor unit size between early and intermediate ages was only 23% for the middle root (Table 2, 1B versus 5B), whereas the decline

was nearly twice as great (42%) for the extreme roots (Table 2, 4B versus 8B). For the slow population during the same period, the decline in motor unit size was even greater for the extreme roots (58%; Table 2, 4B versus 8B), but this was only modestly (1.3 times) more than the decline for middle roots (44%; Table 2, 1B versus 5B). In other words, for the slow motor neurons, the estimated rate of synapse loss is high overall, with only a moderate fractional difference between extreme and middle roots, whereas the rate is considerably lower for the fast population, but with a greater difference between extreme and middle roots.

Rostral vs. caudal motor unit sizes. Figures 5-7 show histograms of the distributions of normalized motor unit tensions for middle roots (upper panels) versus extreme roots (lower panels) for fast and slow populations (left and right panels, respectively) for each age group. In the lower panel, bars are shaded differently for motor units from rostral roots (hatching) and caudal roots (solid). Although there appears to be a trend for motor units from rostral roots to be larger than those from caudal roots, the motor units were indistinguishable in size ($p > 0.1$) for both fast and slow populations from all age groups (see also Table 2). Also, neither combining of data from fast and slow populations for any of the age groups, nor of data from intermediate and late age groups resulted in a significant ($p < 0.05$) rostral versus caudal difference (all comparisons Mann-Whitney U-tests).

Distributions of middle motor unit sizes from normal animals. If a motor neuron's arbor size influences its ability to compete for retention of synapses, the diversity in motor unit sizes should change as synapse elimination proceeds. Our basic measure of diversity was the quartile ratio (the ratio of the values at the first and third quartile of the distribution). An increase in diversity would result in a greater quartile ratio and a decrease would result in a smaller quartile ratio. The quartile

Figure 5. Distributions of normalized motor unit sizes for fast (a,c) and slow (b,d) groups from middle (a,b) and both rostral and caudal extreme (c,d) roots of early age animals. Arrows indicate means. Fast, middle root motor units ($105 \pm 3\%$, mean$\pm$ S.E.M.,$n = 138$) are equal in size to fast, extreme root motor units ($108 \pm 7\%, n = 34, p > 0.2$). Slow, middle root motor units ($106 \pm 6\%, n = 88$) are significantly smaller than slow, extreme motor units ($129 \pm 9\%, n = 31, p < 0.05$). Fast motor units from rostral roots ($109 \pm 9\%, n = 22$) are equal in size to those from caudal roots ($107 \pm 12\%, n = 12, p > 0.2$). Slow motor units from rostral roots ($139 \pm 10\%, n = 20$) are indistinguishable from those from caudal roots ($112 \pm 19\%, n = 11, p > 0.2$).

FIGURE 5

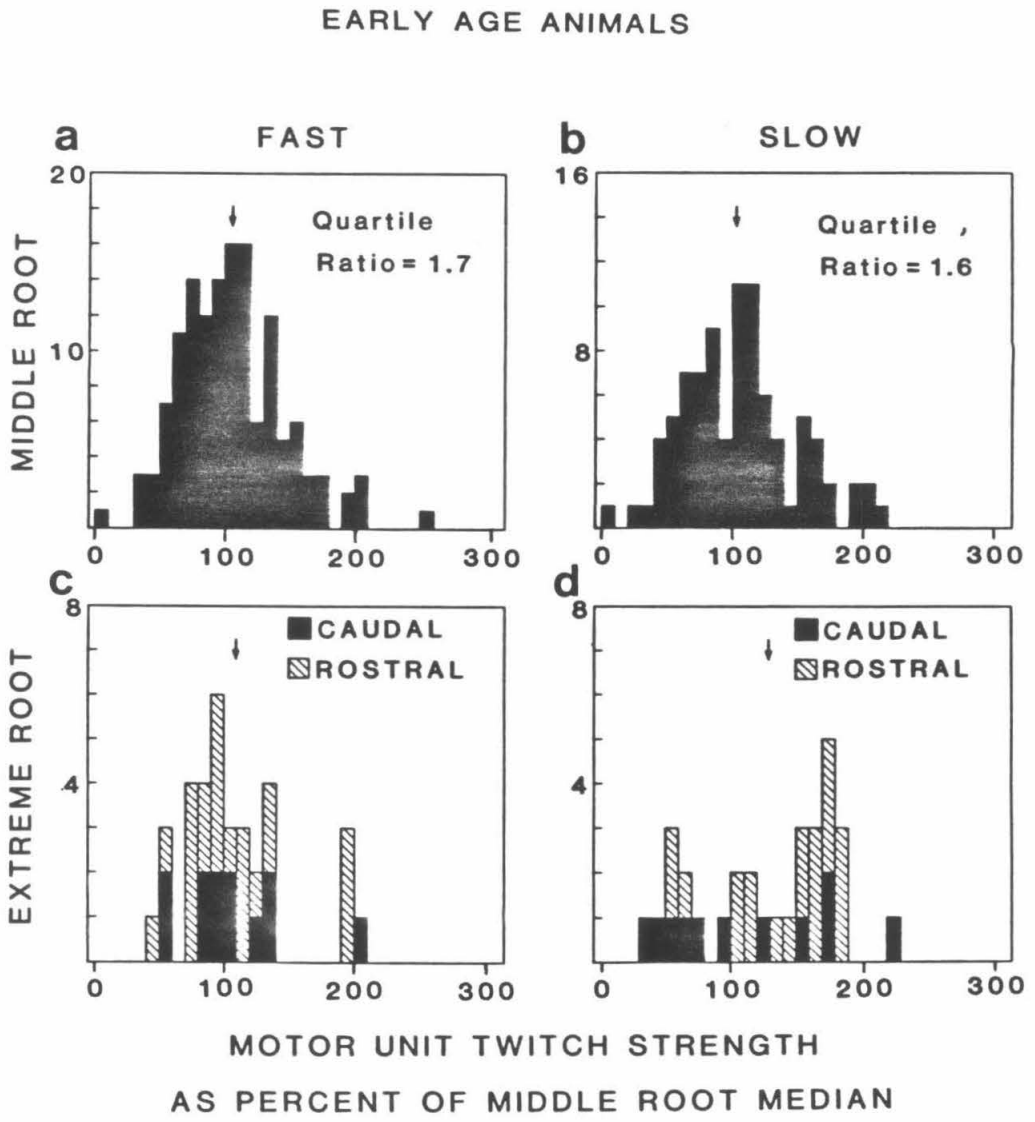

EARLY AGE ANIMALS
a
FAST
b
SLOW
MIDDLE ROOT
EXTREME ROOT
Quartile
Ratio = 1.7
Quartile
Ratio = 1.6
CAUDAL
ROSTRAL
MOTOR UNIT TWITCH STRENGTH
AS PERCENT OF MIDDLE ROOT MEDIAN

Figure 6. Distributions of normalized motor unit sizes for fast (a,c) and slow (b,d) groups from middle (a,b) and both rostral and caudal extreme (c,d) roots of intermediate age animals. Arrows indicate means. Fast, extreme motor units ($79 \pm 5\%$, mean$\pm$ S.E.M., $n = 27$) are significantly smaller than fast, middle motor units ($108 \pm 4\%, n = 101, p < 0.001$) and slow, extreme motor units ($84 \pm 6\%, n = 35$) are significantly smaller than slow, middle motor units ($109 \pm 5\%, n = 115, p < 0.001$). Fast motor units from rostral roots ($93 \pm 10\%, n = 4$) are indistinguishable from those from caudal roots ($77 \pm 5\%, n = 23, p > 0.1$). Slow motor units from rostral roots ($84 \pm 9\%, n = 19$) are equal in size to those from caudal roots ($83 \pm 6\%, n = 16, p > 0.2$).

FIGURE 6

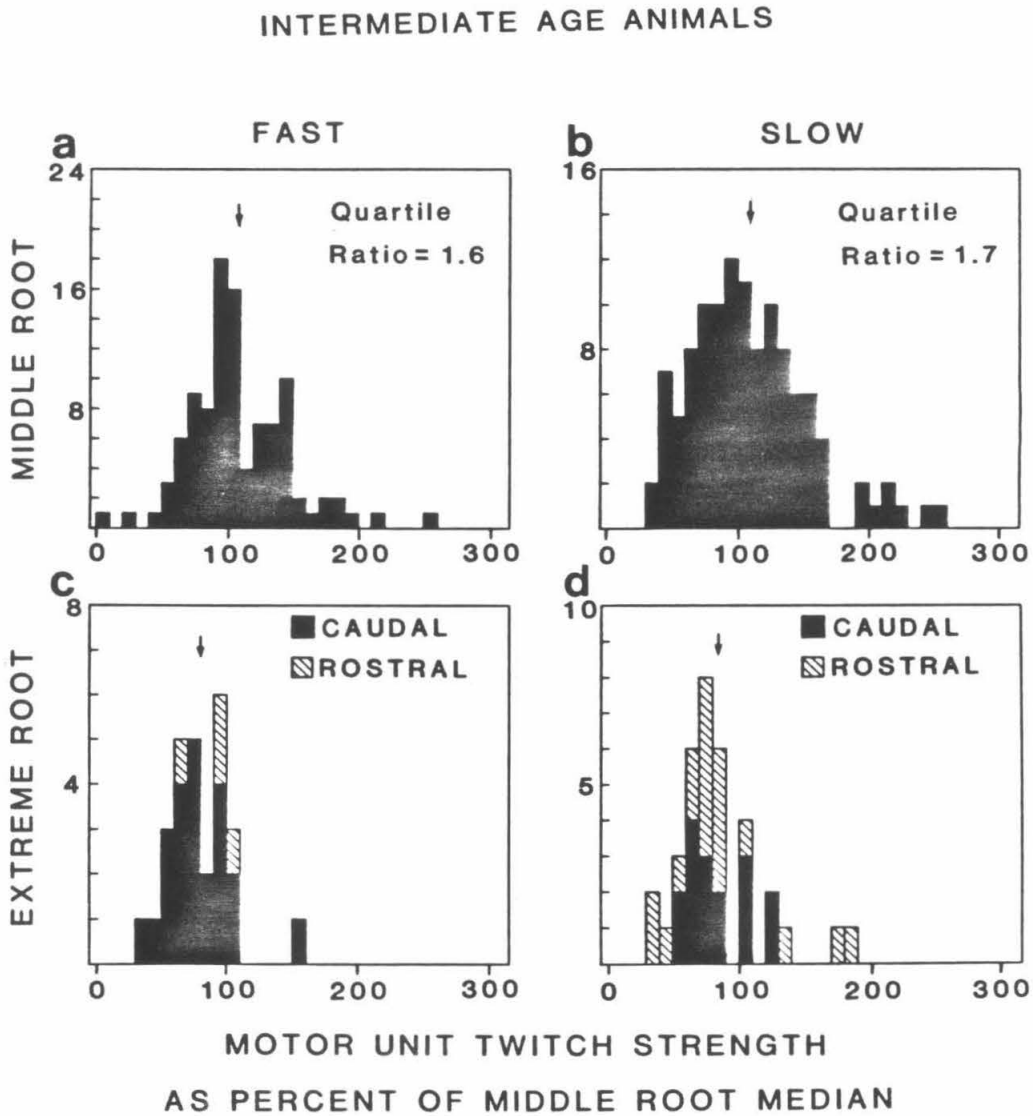
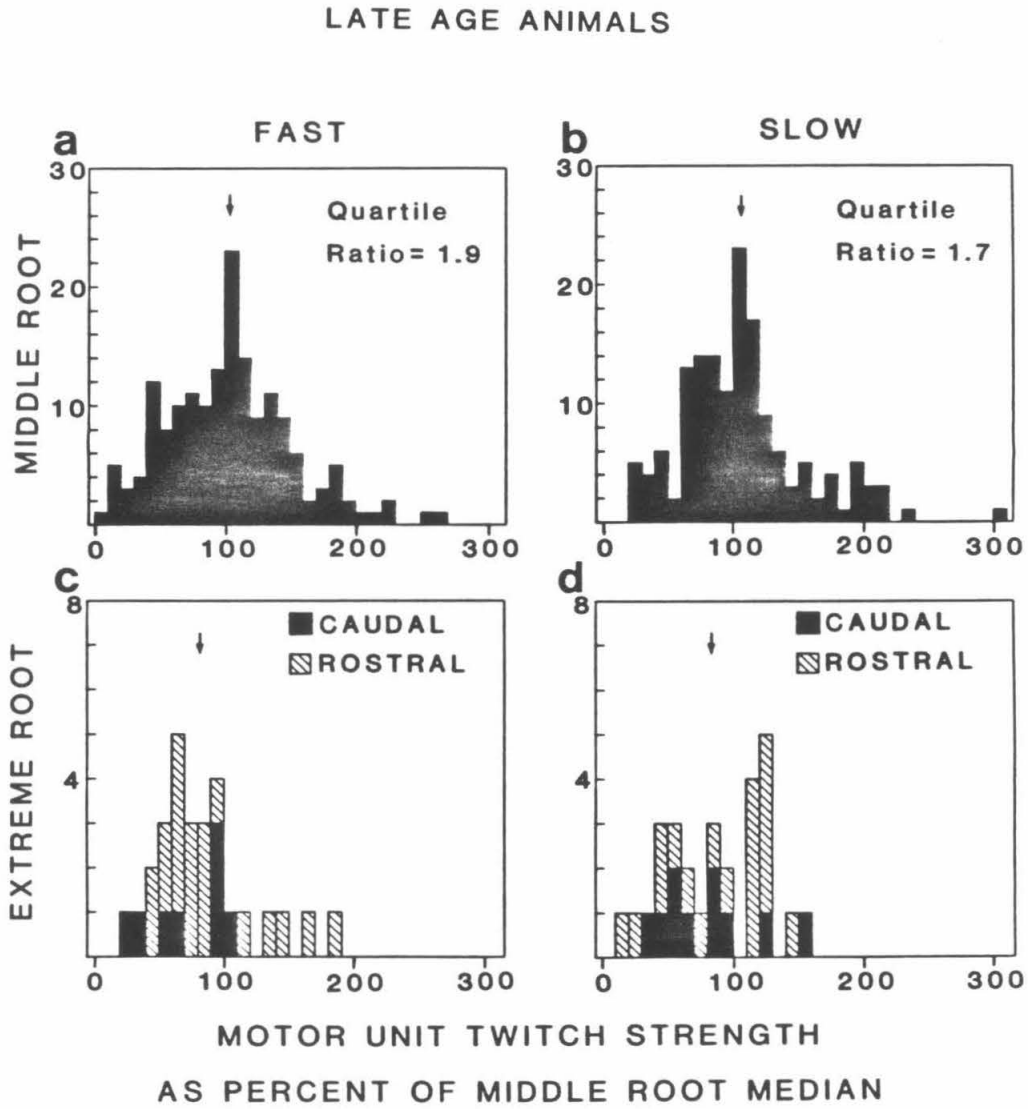

INTERMEDIATE AGE ANIMALS
a
FAST
b
SLOW
Quartile
Ratio = 1.6
Quartile
Ratio = 1.7
MIDDLE ROOT
EXTREME ROOT
CAUDAL
ROSTRAL
c
d
0
100
200
300
MOTOR UNIT TWITCH STRENGTH
AS PERCENT OF MIDDLE ROOT MEDIAN

Figure 7. Distributions of normalized motor unit sizes for fast (a,c) and slow (b,d) groups from middle (a,b) and both rostral and caudal *extreme* (c,d) roots of late age animals. Arrows indicate means. Fast, extreme motor units ($84 \pm 7\%$, mean$\pm$ S.E.M., $n = 28$) are significantly smaller than fast, middle motor units ($103 \pm 4\%$, $n = 167$, $p < 0.05$) and slow, extreme motor units ($86 \pm 7\%$, $n = 28$) are significantly smaller than slow, middle motor units ($107 \pm 4\%$, $n = 152$, $p < 0.05$). Fast motor units from rostral roots ($89 \pm 9\%$, $n = 20$) are indistinguishable from those from caudal roots ($70 \pm 11\%$, $n = 8$, $p > 0.2$). Slow motor units from rostral roots ($90 \pm 9\%$, $n = 18$) are not significantly different from those from caudal roots ($77 \pm 12\%$, $n = 10$, $p > 0.2$).

FIGURE 7


LATE AGE ANIMALS
a
FAST
b
SLOW
Quartile
Ratio = 1.9
Quartile
Ratio = 1.7
MIDDLE ROOT
30
20
10
0
100
200
300
c
d
CAUDAL
ROSTRAL
EXTREME ROOT
8
4
MOTOR UNIT TWITCH STRENGTH
AS PERCENT OF MIDDLE ROOT MEDIAN

ratios for the distributions of normalized motor unit tensions for the middle roots are indicated in the upper right of the appropriate panels in Figures 5-7. Quartile ratios for the fast population distributions were 1.7 at early ages, 1.6 at the intermediate ages, and 1.9 at late ages. For the slow population, the quartile ratios were 1.6 at early, 1.7 at intermediate, and 1.7 at late ages. Thus, the quartile ratios of the distributions change only slightly, and without any trend, for both the fast and slow populations of motor units. Moreover, when compared using the Kolmogorov-Smirnoff test, none of the distributions were significantly different from one another ($p > 0.1$). Accordingly, we infer that arbor size is unlikely to play a major role during postnatal synapse elimination.

Overlap between middle and extreme root motor units at 4-5 days. Twitch responses were measured from whole, ventral roots for 13 normal, early-aged rabbit soleus muscles to determine the degree of overlap of the innervation from the various ventral roots and its dependence on the number of motor units in the extreme (L7 and/or S2) root(s). In all 9 animals in which an extreme root contained 18 or fewer soleus motor axons (range 6 to 18), the whole root twitch tension for S1 was equal to the maximal tension elicited by direct, whole-muscle stimulation (within the 3% error associated with fluctuations in peak tension measurements). This result implies that when an extreme root containing 18 or fewer soleus motor axons was rendered inactive in the TTX experiments described below, virtually all of its synapses were in direct competition with active synapses, and few if any muscle fibers initially lacked active input.

Control- and TTX-implanted animals

Relative motor unit size estimates. Our principal findings are illustrated in Fig. 8, which shows bar graphs of pooled, motor unit tensions from extreme roots of normal

(N), control-implanted (C), and TTX-implanted (T) animals. Solid circles show the mean, normalized tension for individual animals, while the bars show the mean (± S.E.M.) of the pooled distributions.

First, consider the comparison between control-implanted animals and normal animals. There is no significant difference in the normalized motor unit sizes between normal and control-implanted, extreme units for either the fast or slow population at either the intermediate or late age ($p > 0.2$ for all 4 relevant comparisons; see Table 2A, rows 8 vs. 10 and 16 vs. 18). Thus, the implantation of control plugs into extreme roots had no discernible effect on synapse elimination. Therefore, any significant difference in size between TTX-implanted and control-implanted motor unit sizes for any of these groups should be attributable to inactivity induced by the TTX implant.

In contrast to the control implants, Fig. 8 shows that the TTX implants had a major effect on motor unit size. At both intermediate and late ages, and for both fast and slow populations, the normalized size of motor units rendered inactive by TTX-implantation was significantly larger than that of extreme units from both normal and control-implanted animals. In particular, for intermediate-aged TTX-implanted animals, normalized, motor unit sizes from extreme (inactivated) roots were half again larger (45-50%) than the corresponding active, extreme units from control-implanted animals for both the fast population (106% vs. 73%, $p < 0.001$; Fig. 8a; Table 2, 12A versus 10A) and the slow population (137% vs. 91%, $p < 0.001$; Fig. 8a; Table 2, 12A versus 10A). A significant difference was also evident for the late aged animals. The inactivated motor units were about one-third larger (38%) than the control values for the fast population (110% vs. 80%, $p < 0.02$; Fig. 8b; table 2, 20A versus 18A) and more than double the size for the slow population (168% vs. 77%, $p < 0.001$; Fig. 8b; Table 2, 20A versus 18A).

Figure 8. Mean, normalized, motor unit sizes from normal (N), control-implanted (C) and TTX-implanted (T) extreme roots of intermediate (a) and late-age (b) animals. Values for fast motor units are shown at the left side of each figure, slow values at the right. Bars represent the mean ± S.E.M. values for pooled units from each group. Dots represent mean sizes of the extreme root motor units from individual animals. For both fast and slow motor units at both ages, control-implanted units are indistinguishable in size from normal units and TTX-implanted units are significantly larger than control-implanted units. Mean values and significance of comparisons are shown in Table 2. The dot at 172% for the slow population of intermediate-age normal animals represents the mean value from an animal whose extreme root contributed only one slow unit.

FIGURE 8a

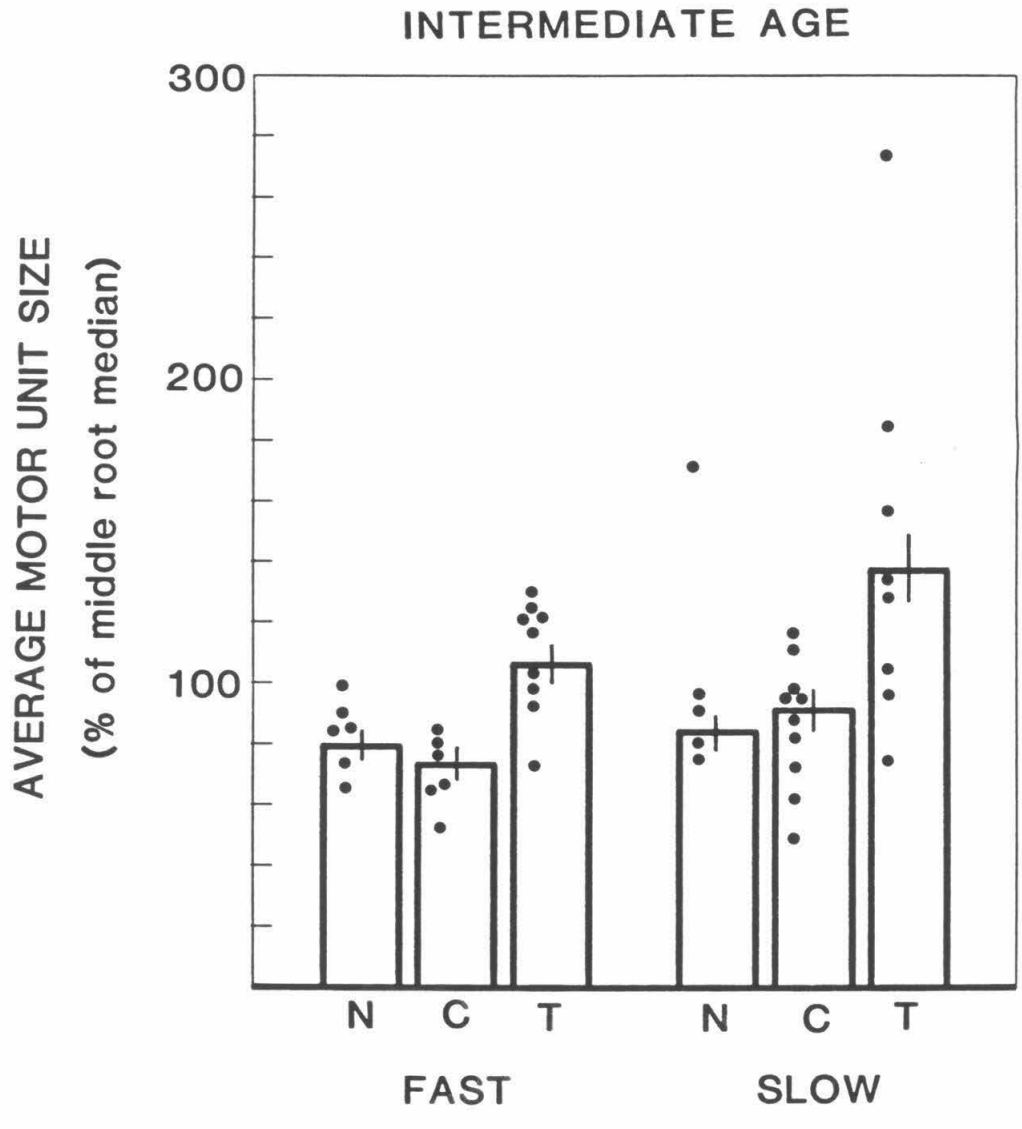
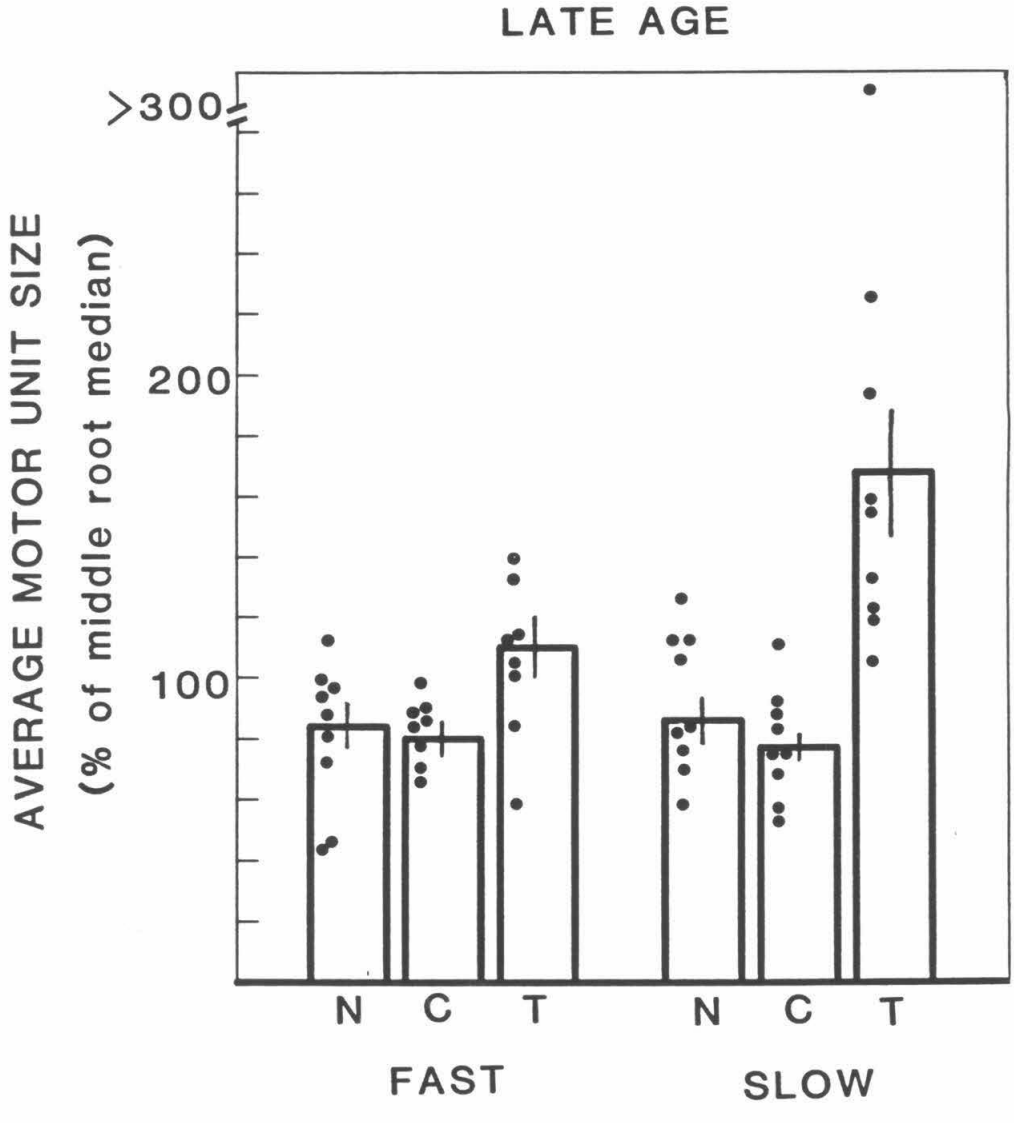

INTERMEDIATE AGE
AVERAGE MOTOR UNIT SIZE
(% of middle root median)
300
200
100
N
C
T
N
C
T
FAST
SLOW

FIGURE 8b


LATE AGE
AVERAGE MOTOR UNIT SIZE
(% of middle root median)
>300
200
100
N
C
T
N
C
T
FAST
SLOW

It is possible to assess the consistency of the effect across individuals by comparing the mean values from the extreme root(s) of individual animals to the overall mean of pooled values from control animals. The mean size from each TTX-implanted animal is larger than the mean size of pooled, control-implanted units in all but 2 cases. The exceptions were: 1) both fast and slow units of one animal from the intermediate group whose extreme (TTX-implanted) root contributed only 1 slow and 1 fast motor unit, and 2) fast units from one animal from the late group whose extreme (TTX-implanted) root contributed only 1 fast motor unit (cf. Fig. 9, animals "n" and "O"). These exceptions are not surprising, given the severalfold range of normalized motor unit sizes at the time of the implant (Fig. 5) and the small numbers of motor units from the extreme roots of these animals; thus, we attribute them to statistical fluctuation. Hence, despite some scatter in the experimental data, our results suggest that the TTX treatment had a consistent effect in each animal subjected to a sustained impulse blockade.

Another important issue is whether the TTX blockade affected all or just a special subpopulation of the motor units within the implanted root. To address this, Figures 9a-d plot the size of each motor unit from the implanted, extreme roots of TTX- and control-implanted animals according to the animal from which they were measured. The mean sizes of pooled motor units from each group are indicated by the dashed lines. The individual motor units cover a wide range of sizes for every group, but sizes are shifted toward larger values for the TTX- versus control-implanted units in all 4 groups (fast and slow units at both intermediate and late ages). In the TTX muscles, there is not only a higher incidence of relatively large motor units, but a lower incidence of relatively small motor units. This result is consistent with the hypothesis that every motor unit from the implanted root was affected by the TTX treatment.

Figure 9. Normalized sizes of individual motor units from extreme roots of control and TTX-implanted animals plotted according to the animals from which they were measured. Mean values for pooled units from each group are represented by dashed lines. Fast units from intermediate age animals are shown in a, slow, intermediate age in b, fast, late age in c, and slow, late age in d. Animals are ordered according to the mean, percentage tension size of fast motor units from their middle root, with lowest values to the left. Intermediate-age, control animals are lettered "a-j", intermediate-age, TTX-implanted animals "k-s", late-age, control animals "A-I", and late-age, TTX-implanted animals "J-T".

FIGURE 9a

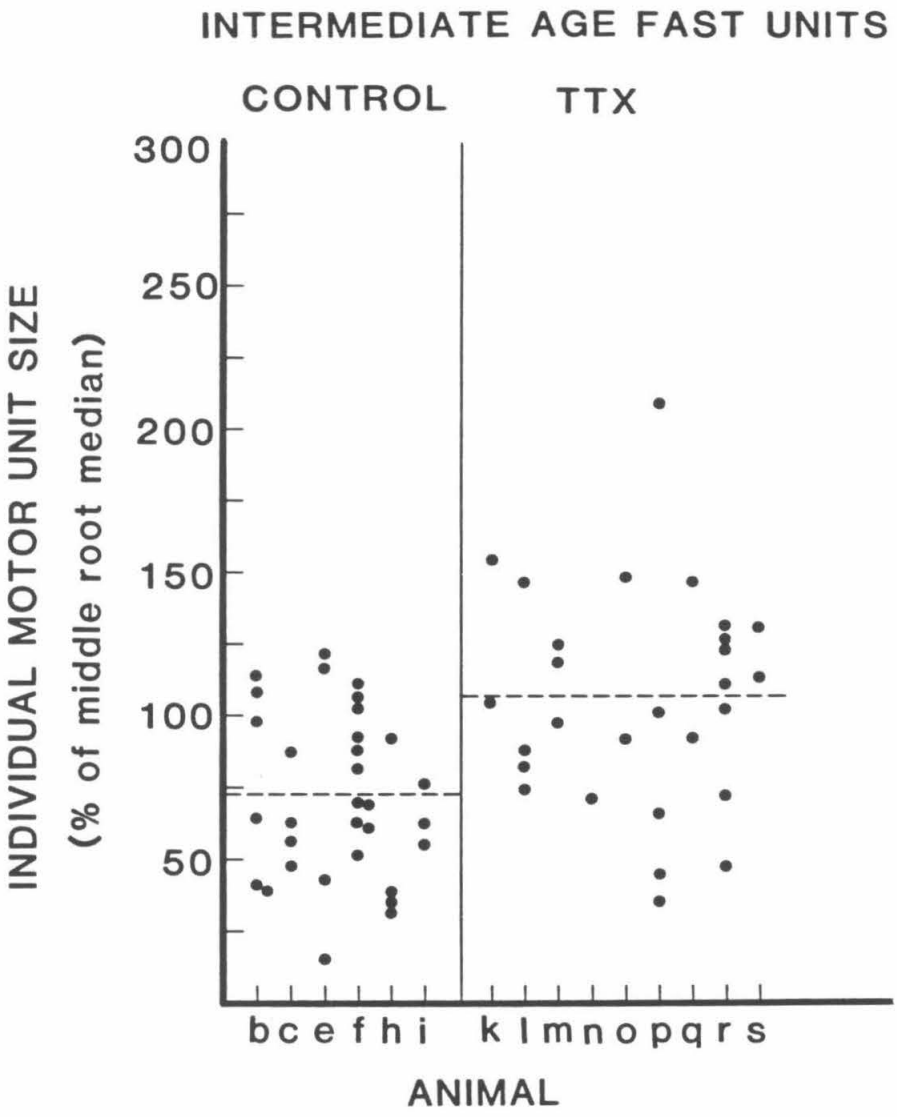

INTERMEDIATE AGE FAST UNITS
CONTROL
TTX
INDIVIDUAL MOTOR UNIT SIZE
(% of middle root median)
300
250
200
150
100
50
b c e f h i
k l m n o p q r s
ANIMAL

FIGURE 9b

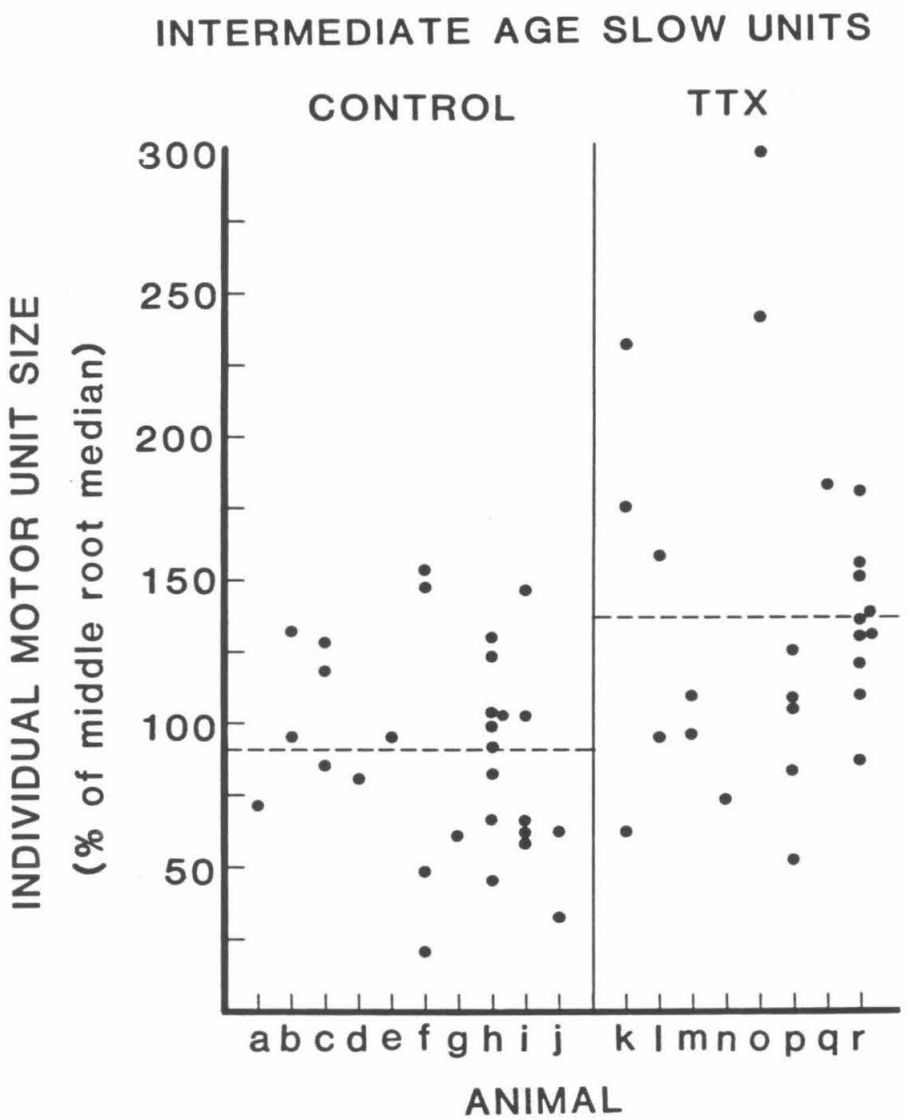

INTERMEDIATE AGE SLOW UNITS
CONTROL
TTX
INDIVIDUAL MOTOR UNIT SIZE
(% of middle root median)
300
250
200
150
100
50
a b c d e f g h i j k l m n o p q r
ANIMAL

FIGURE 9c

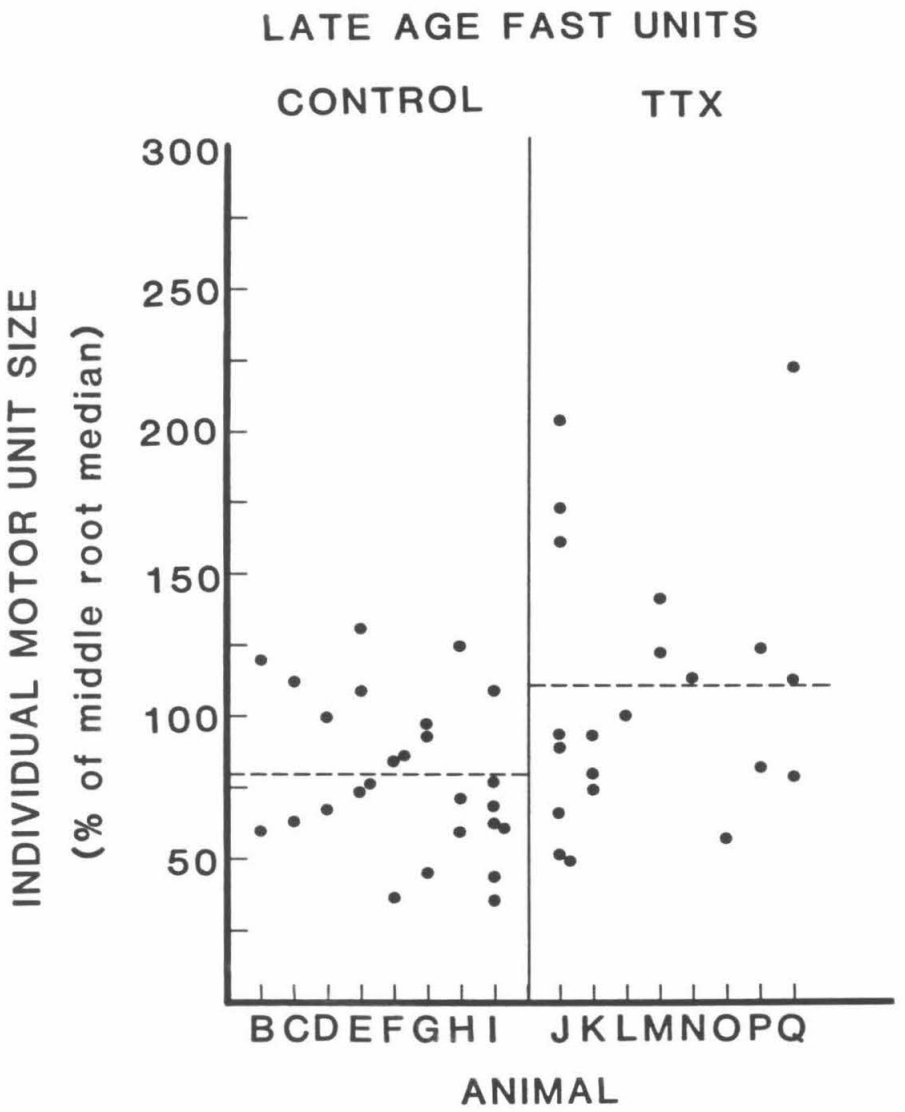
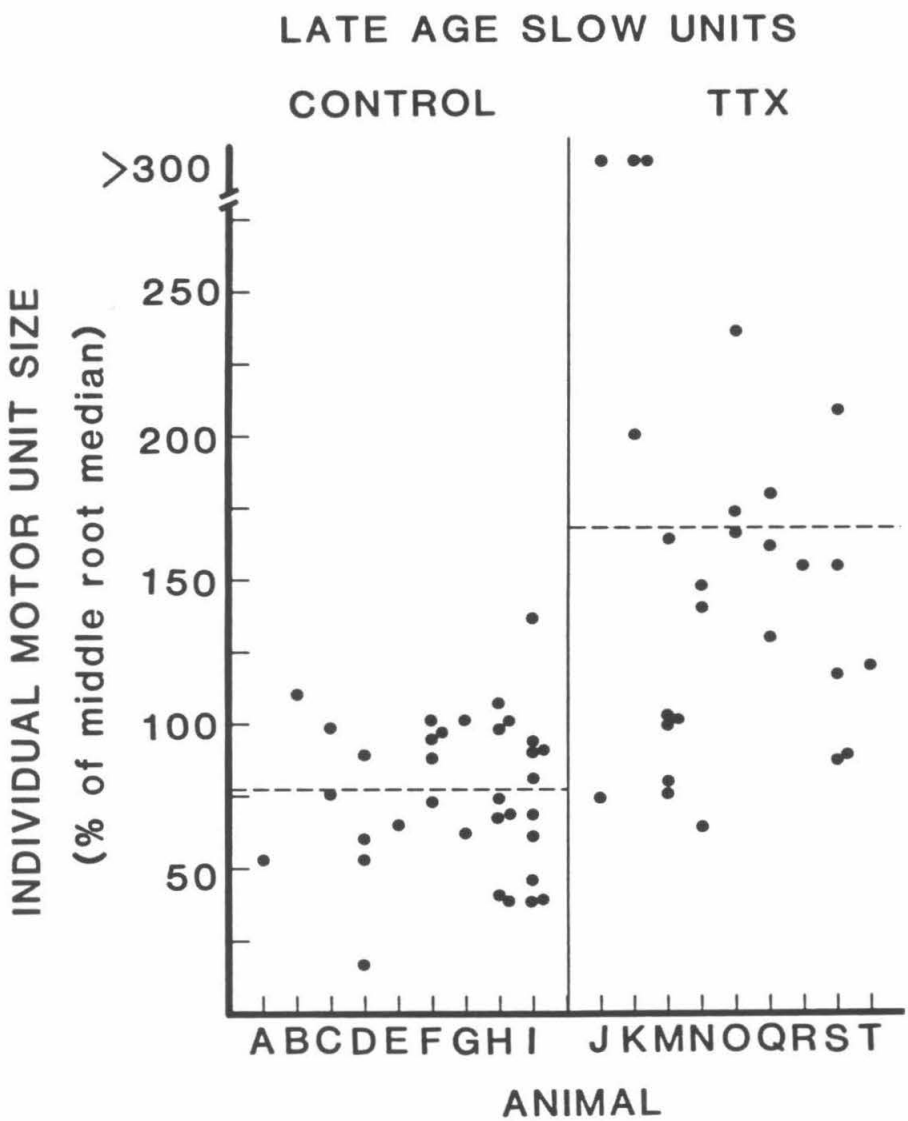

LATE AGE FAST UNITS
CONTROL
TTX
INDIVIDUAL MOTOR UNIT SIZE
(% of middle root median)
300
250
200
150
100
50
B C D E F G H I
J K L M N O P Q
ANIMAL

FIGURE 9d


LATE AGE SLOW UNITS
CONTROL
TTX
INDIVIDUAL MOTOR UNIT SIZE
(% of middle root median)
>300
250
200
150
100
50
A B C D E F G H I
J K M N O Q R S T
ANIMAL

Differential competition versus delayed synapse elimination. Thus far, our analysis has demonstrated a highly significant effect of TTX-blockade on the *relative* size of motor units from blocked and normal roots from the same animals. We can envision 2 general explanations for such an effect. 1) *The competition hypothesis*: differential activity might confer a competitive advantage on inactive synapses. Consequently, inactive motor units (the winners) would end up larger than normal, *and* active motor units (the losers) would end up smaller. By the pure form of this hypothesis, the total rate of synapse elimination would be unaffected, and single innervation of the muscle would be achieved over the normal time course. 2) *The delay hypothesis*: differential activity might delay the process of synapse elimination on those fibers occupied by a mixture of inactive and active synapses. Consequently, both inactive and active motor units would lose synapses at a slower rate than normal (although not necessarily to the same degree), and both inactive and active motor units would be larger than normal as long as polyinnervation persisted. In addition, the achievement of single innervation would be delayed. Again, considering the pure form of this hypothesis, differential activity would not affect the ultimate outcome in terms of which particular synapses were eliminated, only the rate. We used 3 independent approaches to distinguish between these hypotheses.

Our first approach was to determine what effect the TTX blockade had on the absolute size of motor units from the middle roots, as assessed by percentage tension measurements. We found that the mean percentage tension from the middle root was consistently smaller for the TTX animals than for either the control or the normal animals, a result that supports the competition hypothesis. For example, the mean size of slow motor units from intermediate-aged, TTX-implanted animals was 16% smaller than that from normal animals ($1.6 \pm 0.2\%$ versus $1.9 \pm 0.3\%$, Table 2, 11B versus 5B) and 30% smaller than that from control animals ($2.3 \pm 0.3\%$, Table

2, 9B). Similar results were found for every one of the other 6 comparisons, involving fast motor units at the intermediate age and both types at the late age. On average, middle root motor units were 17% smaller in the TTX cases than in normal and control muscles. This difference is highly significant ($p = 0.001$, Mann-Whitney U-test based on individual animal means normalized to the overall mean for normal and control animals combined).

Although these differences were small, they actually exceed the amount predicted on the basis of the numbers of fibers in extreme and middle roots and the magnitude of the inactivity effects on the extreme roots. On average, the TTX blockade involved fewer than 10% of the total number of soleus motor axons. Hence, if the inactive motor units ended up about 60% larger than normal (see above), then by the pure competition hypothesis, the active motor units should end up less than 6% reduced in size. The fact that our measurements yielded a difference of 17% could reflect a contribution from individual variability in percentage tension values. An alternative possibillity is an actual acceleration of the overall rate of synapse elimination, in addition to a competitive advantage for inactive synapses.

Our second basis for distinguishing between the competition and delay hypotheses was an assessment of the degree of polyinnervation in late-age control and TTX cases. If the size difference between motor units from control-implanted and TTX-implanted roots were due to delayed synapse elimination in the TTX-implanted animals, there would be greater polyinnervation in their muscles. We used a histochemical, silver-staining technique to assess the degree of polyinnervation in muscles from late-age, TTX- and control-implanted animals (see Methods). A few polyinnervated fibers were encountered both in control cases (2 definite and 1 questionable out of 900 endplates examined in 9 muscles) and TTX cases (4 definite and 1 questionable out of 1100 endplates in 11 muscles). We

conclude that fewer than 0.5% of the fibers were multiply innervated and that there was no significant difference between the two groups.

We estimated how much of an increase in polyinnervation in the TTX-implanted muscles would be necessary in order for the delay hypothesis to account for the observed difference in normalized motor unit size between control- and TTX-implanted roots. At the late age, motor units from TTX-implanted roots were about 80% larger than control-implanted (average of 118% and 38% for the slow and fast populations). Only about 6% of the motor axons were blocked, however (4.4 on average out of an estimated 70 soleus motor axons). The product of these two numbers (6% and 80%) is about 5%, which represents the incidence of polyinnervation predicted by the delay hypothesis. This is an order of magnitude greater than the observed incidence, which argues strongly against the delay hypothesis.

Another measure of polyinnervation is tension overlap. The greater the degree of polyinnervation, the more overlap among motor units. We assessed the overlap between middle and extreme roots in normal and control versus TTX-implanted animals, by calculating the percentage of muscle fibers innervated by the extreme root that were not in addition innervated by the middle root. By this measure, termed percentage-exclusive innervation, larger values correspond to less overlap (cf. Callaway et al., 1987 for further details). We have concentrated on the fibers innervated by the extreme root because these are the fibers at which elimination should be slowed in TTX-implanted animals, according to the delay hypothesis. At the intermediate age, the percentage-exclusive innervation was $34 \pm 8\%$ (mean $\pm$ S.E.M.) for TTX-implanted versus $19 \pm 8\%$ for normal and control animals, a difference that was statistically significant ($p < 0.025$, Mann-Whitney U-test). This result supports the competition hypothesis and argues against the delay

hypothesis. For late-age animals, the amount of overlap was quite small. Percentage exclusive innervation was 97% to 100% for muscles from both TTX- and control-implanted animals. The slight variations are attributable to error from noise in our measurements, and the results therefore indicate that residual polyinnervation was minimal at this stage, in agreement with the silver-stain results.

Percent synapses lost by active and inactive motor units. Our evidence indicates that the TTX treatment slowed the rate of synapse elimination among motor neurons from the implanted root, but it did not halt the process altogether. To estimate the change in rate of synapse elimination, we compared percentage tension values at different ages. The decline in motor unit size by fast, inactive, TTX-implanted motor neurons from intermediate-aged animals was only 27% (6.7% to 4.9%; Table 2, 4B vs. 12B) compared with 42% from both normal and control-implanted extreme roots of the same age (Table 2, 4B vs. 8B and 10B). This suggests that the TTX blockade reduced the rate of synapse elimination by about one-third (36%) among inactive motor neurons. For the slow population, the decline in motor unit size for TTX-implanted motor neurons from intermediate-aged animals was 45% (Table 2, 4B vs. 12B) compared with 58% and 53% for same aged normal and control-implanted extreme roots, respectively (Table 2, 4B vs. 8B and 10B). This corresponds to a 22% reduction in the rate of elimination among inactive motor neurons.

The rate of synapse elimination, once the TTX blockade wore off, can be estimated by comparing motor unit sizes for TTX-implanted roots examined at intermediate and late ages. For the slow population, average motor unit size declined by 48% for TTX-implanted roots between intermediate and late ages (Table 2, 12B vs. 20B). Similar declines took place for normal and control extreme roots (46% and 64%, respectively; Table 2, 8B vs. 16B and 10B vs. 18B). For

the fast population, the decline was 45% for TTX-implanted roots (Table 2, 12B vs. 20B) and 36% and 31%, respectively, for normal and control extreme roots (Table 2, 8B vs. 16B and 10B vs. 18B). Although this suggests a slightly greater rate of elimination for fast, TTX-implanted neurons while they were active, there is no significant difference between the more sensitive, normalized tension values for the fast populations of TTX-implanted motor units at intermediate versus late ages (106% vs. 110% of median, $p > 0.2$; Table 1, 12A versus 20A).

Possibility of repetitive muscle fiber contraction. We were concerned about whether the increased tension produced by previously inactivated motor units might have been an artifactual consequence of repetitive firing. Ribchester and Taxt (1983) reported that endplate potentials at muscle fibers lacking active inputs tend to be prolonged, and the inputs to such fibers can fire repetitively. Either of these effects could lead to repeated contraction of the muscle fiber, resulting in greater than normal tension during a twitch response. They observed that for inactive motor units in muscles with the majority of the fibers inactive, the twitch rise-times were slower than normal, presumably because of repeated contraction of muscle fibers. Neither repetitive firing nor prolonged endplate potentials were reported at muscle fibers with even small amounts of active input. These phenomena are therefore unlikely to occur in our TTX-implanted animals, since virtually every muscle fiber has at least one active input at the outset of the experiment, and in the late-age animals, all of the inputs had been active for at least 6 days at the time of the assay. Also, if muscle fibers had fired repeatedly during twitch responses for the TTX-implanted motor units, their rise times would be expected to be slower than normal (as observed by Ribchester and Taxt, 1983). In actuality, twitch rise-times for TTX-implanted motor units were slightly faster than for normal or control-implanted motor units (data not shown). It is therefore unlikely that repetitive

contraction of muscle fibers contributed to the increased size of twitch responses from inactivated motor neurons in the TTX-implanted animals considered above. For major block animals, however, this is a stronger possibility (see below).

TTX-implanted, major-block animals

For three TTX-implanted animals analyzed at the intermediate ages, a large fraction of the soleus motor units was inactivated (see Table 1), either because of a branch splitting from the S1 spinal nerve and joining S2 proximal to the TTX implant (2 cases) or because of an unusually large number of soleus motor axons in S2 proper (1 case). In these cases, a substantial percentage of the soleus muscle fibers had all of their inputs inactivated. Based on previous studies, synapse elimination should be delayed in these totally inactive fibers (Brown et al., 1981; Thompson et al., 1979; Duxson, 1982; Chapter 2). If muscle inactivity had no other effects, synapse elimination would be expected to proceed in the remaining fibers, with active synapses at a disadvantage when competing with inactive ones. By this logic, inactive motor units should be larger than normal, and active motor units should be the same as or smaller than normal. The actual results from these animals suggest that this is indeed the case for the slow motor units but, surprisingly, not for the fast population.

The twitch responses from combined stimulation of all blocked axons were 80-95% of maximal direct tension. The responses from active roots were estimated to be 50-90%. Thus, we estimate that 50-70% of the soleus motor axons were inactivated. About 20-50% of the muscle fibers had no active inputs; 5-20% had only active inputs, and the remainder (45-60%) had overlapping active and inactive inputs.

For the fast population, the TTX blockade led to an increase in motor unit size for active as well as inactive motor units. Specifically, average motor unit size was 7.4± 1.1% of maximum for active, fast motor units and 6.6±0.4% for inactive, fast units, both of which are significantly larger than middle root motor units from normal, intermediate-aged animals (5.0±0.5%, $p < 0.025$).

For the slow population, the TTX blockade resulted in inactive motor units that were twice as large as normal (3.8±0.7% versus 1.9±0.3%, $p < 0.01$). The active motor units, on the other hand, were not significantly larger than normal (2.6±0.6% versus 1.9±0.3%, $p > 0.1$). The difference between active and inactive units was not significant when based on the combined means from all 3 animals. However, there is clearly a significant difference between active and inactive motor units when comparing the intra-animal distributions in 2 of 3 cases: 5.2±0.4% (19 units) versus 3.5±0.3% (4 units), $p < 0.05$ and 3.1±0.3% (14 units) versus 1.4±0.4% (13 units), $p < 0.001$). Therefore, it appears that the fast and slow populations were affected differently by the activity block of the same percentage of motor axons; the results from the fast population were similar to those expected from activity block of the entire neuronal population, while the results from the slow population were more similar to those for minor block animals.

Discussion

In this study we have tested for: 1) differences in the degree of polyinnervation and rate of synapse elimination for fast versus slow muscle fibers; 2) a correlation between axonal arbor size and the number of synapses lost by motor neurons of a given contractile type; 3) spatial gradients in the extent of synapse loss for motor neurons from different spinal positions; and 4) differences in competitive ability between active and inactive motor neurons. Our major findings relate to the last two issues (items 3 and 4 above), but we will start by discussing the first two, as they can be dealt with expeditiously.

Normal animals.

Axonal arbor size. Previous reports have noted an initial increase in diversity of motor unit sizes during the first two postnatal weeks and have suggested accordingly that terminals belonging to large motor units might be at a competitive advantage over those from smaller motor units (Gordon and Van Essen, 1981; Van Essen, 1982). On the other hand, it has been suggested both on theoretical grounds (Smalheiser and Crain, 1984) and based on motor unit size estimates that larger motor units might be at a disadvantage because of the metabolic constraints of maintaining a larger axonal arbor.

Our results do not support either of these hypotheses. We found no indication that arbor size influences the competitive ability of motor neurons during synapse elimination in the rabbit soleus. There are only small differences between distributions of middle motor unit sizes for different aged animals based on their quartile ratios, and the distributions do not vary significantly in any way, including diversity, as judged by the Kolmogorov-Smirnov test. If arbor size did play a role,

the diversity of motor unit sizes would be expected to change as synapse elimination progressed.

The difference between the present results and those from previous studies is most likely due to our having separated fast and slow motor unit populations in this study. Without this separation, our results also show an increase in diversity, since fast units averaged only 1.9 times larger than the slow units at early ages, but the difference increased to 3.1 times at late ages. As discussed below, this change in relative size probably represents, at least to some degree, a difference in rate of synapse elimination for fast versus slow muscle fibers.

Results from TTX-implanted animals provided an additional test of this issue. TTX treatment created a subpopulation of soleus motor units that was abnormally large (about 50% increase) at 8-9 days. After the block wore off in the long-term experiments, these abnormal units were left to compete with relatively normal-sized units, and a normal activity pattern had presumably been restored. If arbor size were important, the larger than normal units would be expected to preferentially increase or decrease in size relative to the remainder of the units. In contrast to this prediction, there was no significant difference in mean, normalized, motor unit size between TTX-implanted motor units analyzed at 8-9 days and those whose activity had resumed at 8-9 days and were analyzed at 14-15 days. The results from the normal animals, however, probably give the best indication of the upper limit of any effect of arbor size, since the analysis from TTX animals is probably not as sensitive to small effects.

Fast versus slow muscle fibers. Our results are consistent with the suggestion of Gordon et al. (in preparation), that the extent of polyinnervation at birth is greater for the slow population of rabbit soleus muscle fibers than for the fast population.

We extend their observations from newborn (1-5 day) and 11-18 day age groups to include 4-5 day and 8-9 day animals. Both sets of results suggest either that fast fibers are initially contacted by fewer motor neurons than are slow fibers, or that synapse elimination begins earlier for the fast population.

This interpretation is dependent on the assumption that the ratio of the tension generated by individual fast and slow muscle fibers does not change substantially from birth to 15 days. To the extent that this assumption is correct, the ratio between average motor unit size in polyinnervated muscles to that in singly innervated (late age) muscles will reflect the number of synapses per muscle fiber. If the assumption is incorrect, this ratio will reflect a combination of polyinnervation and changes in muscle characteristics. That our calculations are not far off the mark is supported by the good agreement between our estimated value of 1.75 synapses per muscle fiber at intermediate ages (average for fast and slow) and the estimate of 1.7 synapses per fiber obtained from intracellular recording in rabbit soleus muscles of the same age (Soha et al., 1987).

Based on the present evidence, the rate of synapse elimination appears to be greater for the slow than for the fast population of muscle fibers, but with both populations reaching the singly innervated state at about the same time. Soha et al. (1987) reached a similar conclusion about the synchrony of onset of single innervation in fast and slow fibers.

Rostral versus caudal motor neurons. The possibility that the extent of synapse elimination by motor neurons is dependent on their spinal position was first raised by Miyata and Yoshioka (1980), who suggested on the basis of an indirect assay that rostral motor neurons lose many more synapses than caudal motor neurons in the rat soleus. Subsequent direct measurements of motor unit sizes have revealed

no rostral versus caudal bias for the rat soleus (Thompson, 1983b; Gordon and Van Essen, 1983), mouse soleus (Fladby, 1987), or the rabbit soleus (Gordon and Van Essen, 1983). Our results are consistent with these, in that we do not find any marked difference in the extent of synapse elimination for motor neurons from the rostral versus caudal extremes in the rabbit soleus motor pool. Although there was a modest tendency for rostral motor units to be larger than caudal units (average of 13% from comparisons for each fiber type at all 3 ages), none of these differences was significant. Our results extend previous studies by showing that the conclusion applies to both fast and slow populations of motor units.

Extreme versus middle motor neurons. Unexpectedly, we found that during normal maturation, motor unit tensions decline more extensively for inputs from extreme spinal roots than from the middle root. This difference exists for both the fast and slow populations of motor neurons and for both rostral and caudal extremes, and it develops during a brief window between early (4-5 day) and intermediate (8-9 day) ages.

Conceivably, this change might reflect a difference in tension generated per fiber among muscle fibers innervated by the extreme versus middle root, rather than a difference in relative number of synapses lost. Such a change could presumably result from: 1) selective loss of terminals from fibers according to contractile strength; 2) selective loss at fibers whose position in the muscle does not allow them to contribute effectively to the measured tension; or 3) to a change in contractile strength of fibers according to the neurons that innervate them. These possibilities all seem quite unlikely, given the degree of overlap between extreme and middle root innervation at early ages (complete overlap) and intermediate ages (about 80% overlap for fibers innervated by the extreme root), coupled with the observation that extreme units generate at least as much tension as middle units at the early age.

Although there is extensive overlap for fibers innervated by extreme roots, the majority of the muscle fibers are innervated exclusively by the middle root at both ages. Therefore, the selective loss hypotheses (1 and 2 above) would necessitate that the extreme neurons originally innervated both weak and strong fibers (whereas middle root neurons only occasionally innervated weak fibers), and that inputs to the weaker fibers were preferentially maintained. However, the average motor neuron lost fewer than half of its synapses from early to intermediate ages. Hence, for the difference observed at the intermediate age to be due to selective maintenance at weak fibers, there should have been a motor unit tension difference at the early age that was at least half as large as at the intermediate age. Our results indicate that no such difference was present.

The possibility of a substantial change of contractile strength over a period of only 4 days (hypothesis 3) seems unlikely. In addition, such a change would have to be dependent on the spinal position of the neurons innervating the fiber to account for our result. Given the high degree of overlap at fibers innervated by the extreme root at the intermediate age, the fibers would receive conflicting information as to the origin of their innervation. Hence, we favor the interpretation that there is an actual difference in synapse loss between extreme and middle roots.

Whatever the nature of the disadvantage to extreme motor neurons, it is apparently manifested primarily during the early stages of synapse elimination. One explanation for this could be that an initial disadvantage to extreme motor neurons is later offset by a relative advantage gained by a smaller arborization. However, we do not favor this interpretation since, as noted above, arbor size does not appear to influence synapse loss.

A second possibility is that by 8 to 9 days the fate of each synapse has already

been determined. For example, a single synapse may already occupy the majority of the endplate region, placing it at an irrevocable advantage, wherupon factors such as spinal position, arbor size, and activity would no longer be able to influence the outcome of the competition strongly. We know of no evidence bearing directly on this hypothesis, although it is consistent with the report that inputs to the neonatal rat's lateral gastrocnemius muscle, which are more likely to be eliminated, evoke relatively small endplate potentials (Bennett and Lavidis, 1984).

We can envision two types of mechanism that might account for differential synapse elimination according to spinal position. The first involves activity-dependent effects on motor unit size. Based on our finding that inactivity increases a motor neuron's competitive ability, higher activity levels might be expected to confer a disadvantage. Therefore, to account for our results in terms of an activity difference, it would be necessary to suppose that extreme motor neurons were more active than those in the middle. We know of no evidence for differential activity of middle and extreme motor neurons, although such differences might result from variations in afferent connections to extreme and middle motor neurons or from variations in cell body size.

The second possibility, more appealing to us, involves chemospecific matching between motor neurons and muscle fibers. Suppose, for example, that there were positional differences in the spinal cord, such that soleus muscle fibers were better matched to neurons from the middle of the soleus motor pool than to those from either extreme, and that this differential matching were manifested during synapse elimination rather than during synapse formation. Alternatively, there might be gradients in the muscle as well as in the motor neuron pool. For instance, each motor neuron might be optimally matched to muscle fibers in a restricted portion of the muscle, and the rostro-caudal spinal axis might map onto a particular axis

(e.g., *dorso-ventral*) in the muscle. If the gradient in the muscle were shallower than the gradient in the spinal cord, then motor neurons from extreme positions would be optimally matched with fewer muscle fibers than motor neurons from middle positions. Again, supposing that the differential matching were manifested during synapse elimination, this would lead to greater synapse loss by extreme motor neurons.

Evidence for some form of segmental specificity between nerve and muscle has come from reinnervation experiments, in which intercostal muscles are preferentially reinnervated by preganglionic autonomic neurons from specific spinal segments (Wigston and Sanes, 1982; 1985) or in which a topographic map is restored by reinnervating spinal motor neurons (Laskowski and Sanes, 1987b). It is not clear from these studies, however, whether the modest segmental biases revealed at the time of the assay reflect differential synapse formation or differential synapse elimination. More direct evidence for a role of differential synapse elimination in the sharpening of topography has come from studies of segmental innervation patterns during normal maturation in the rat gluteus (Brown and Booth, 1983) and rat lateral gastrocnemius muscles (Bennett and Lavidis, 1984; Bennett et al., 1986; Bennett and Ho, 1988). These observations suggest that the synapses from a single motor neuron initially make a large number of connections over a large but still crudely appropriate topographic area, and as synapse elimination proceeds, the more topographically appropriate connections tend to win the competitions on the individual muscle fibers. On the other hand, not all muscles are topographically organized. The soleus muscle apparently lacks segmental topography in both mouse (Fladby, 1987) and rat (Thompson et al., 1984); it is unclear what the pattern is in the rabbit, which has many times more muscle fibers. Thus, while the aforementioned findings are supportive of the matching hypothesis, they are

consistent with either the single-gradient or double-gradient hypothesis.

Whatever the developmental mechanism might be, it is of interest to speculate on the functional significance of the differential loss of synapses according to spinal position. It seems unlikely that the greater synapse loss by extreme motor neurons represents a simple error correcting scheme, since extreme motor units remain substantial in size and overlap extensively with the distribution of middle motor units. If connections from extreme neurons were functionally inappropriate, differential cell death or a much greater difference in extent of synapse loss (as suggested by Bennett et al., 1986) would be needed for effective error correction. Alternatively, there might be a functional advantage related to neuronal activity patterns as outlined in the following sections. However, it also seems quite plausible that this differential synapse loss may have no direct adaptive significance for the soleus muscle. Rather, it may be an epiphenomenon that reflects a general set of developmental interactions needed to attain appropriate matching between nerve and muscle throughout the body.

Activity

Competition between active and inactive synapses. We have tested the influence of activity on the ability of neuromuscular synapses to compete for occupancy of endplates during neonatal synapse elimination. In the paradigm used, active and inactive synapses were pitted directly against one another, at endplates on active muscle fibers, by inactivating a small fraction of the soleus motor axons. There were four key findings from these experiments. 1) Motor unit tensions from TTX-inactivated roots were approximately 50% larger than those from control-implanted or normal extreme roots. 2) This effect appeared over a period of 4-5 days of differential activity, and it persisted even when activity in the initially inactive root

was restored. 3) The increase in average tension from inactive motor units was coupled with a small but significant decrease in average tension of motor units from the active middle root. 4) There was no discernible increase in the incidence of polyinnervation as assessed by physiological tension overlap measurements or by histological analysis of silver-stained terminals.

We have considered a variety of potential sources of error or systematic bias in the degree to which our twitch tension assay reflects actual motor unit sizes. Our findings are not attributable to misclassification of motor unit, twitch types, repetitive firing of muscle fibers, or differences in the under-representation of maximal twitch responses for different muscles. It is also highly unlikely that the TTX treatment simply caused a delay in synapse elimination among muscle fibers innervated by the implanted root.

We conclude that differential activity had a direct effect on synaptic competition, leading to a lower rate of synapse loss among inactive motor neurons, that was offset by an increased rate of synapse loss among active motor neurons. Inactive motor units ended up about 50% larger than normally active motor units from control animals. Recall, however, that this increased competitiveness was provided to motor neurons which initially were at a slight disadvantage owing to their origin from an extreme spinal root. For slow motor units, the effect was large enough that the inactive units actually became significantly larger than the active units from from the middle root ($p < 0.01$ for intermediate and $p < 0.005$ for late age animals, Mann-Whitney U-test). For fast motor units, the effect was only sufficient to restore the extreme motor units to approximate parity with the middle root units.

There are two plausible explanations that might account for the greater effect of differential activity on slow motor units. First, the overall rate of synapse

elimination was greater for slow than for fast motor units (58% versus 42% for extreme roots between early and intermediate ages; cf. Table 2). If differential activity elicited a fractional change rather than an absolute rate change, then a greater effect on slow motor units would be predicted. Second, average activity levels are reportedly about twice as great for slow as for fast motor units (Navarette and Vrbova, 1983). Consequently, TTX-inactivation would lead to a greater change in activity for the slow motor units, which, in turn, might result in a greater effect on motor unit size.

Four studies have reported evidence suggesting an advantage to more active synapses, contrasting with the conclusions of the present results. One of these studies involved differential activity during normal neuromuscular development (Ridge and Betz, 1984); two involved reinnervation of adult muscle (Ribchester and Taxt, 1983; Ribchester, 1988); and the last involved a tissue culture preparation (Magchielse and Meeter, 1986).

The closest paradigm to our own was the study of Ridge and Betz (1984), who used electrical stimulation to increase the activity of one of the two nerves innervating the lumbrical muscle in neonatal rats. They reported that motor units from stimulated nerves were slightly larger than those from unstimulated nerves. However, these differences were not statistically significant when tested by standard procedures (Student's T-test or Mann-Whitney U-test). They claimed that the result was significant when analyzed by another procedure, but the statistical validity of this alternate procedure is questionable in our opinion. [1] While we are

[1] In their published report, motor units whose sizes were expected to be different than normal (from the "minority" populations mentioned above) were compared to all the other units from the same animal (including the contralateral muscle), whose sizes were expected to be relatively normal. Thus, numerous ratios were obtained

not convinced by their arguments, it is nonetheless possible that their interpretation is indeed correct. If so, the difference between their results and ours might be related to the species used (rat versus rabbit), the particular muscle (lumbrical versus soleus), or to the means of achieving differential activity (high versus normal activity in their experiments; normal activity versus inactivity in ours).

One particularly noteworthy difference between development in the rat lumbrical and rabbit soleus muscles is that the number of muscle fibers in the lumbrical nearly doubles during the period of synapse elimination (Betz et al, 1979), while there is a constant number for the rabbit soleus (Bixby and Van Essen, 1979). Thus, during the period when Ridge and Betz (1984) invoked differential activity, there is normally no net loss of synapses; rather, each lumbrical motor neuron maintains a roughly constant number of synapses because synapse loss is offset by formation of synapses on the newly generated fibers (Betz et al., 1979). The hypothesis that activity is an advantage for synapse formation but a disadvantage during synapse elimination is therefore consistent with both the present results and those of Ridge and Betz (1984). According to this hypothesis, there would be little net effect of differential activity on motor unit size during early stages of synapse elimination in the lumbrical (as Ridge and Betz, 1984 observed) because of the

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from a small sample of "minority" units; for example, the 4 units from the animals in which a minority of the motor neurons were stimulated yielded 23 ratios. The pooled ratios for units expected to be larger than normal were then compared to ratios expected to be smaller. The result was deemed significant, based on statistical tests on the pooled ratios (U-test or T-test), apparently without regard for the need for samples in such tests to be obtained independently. Because the numerous ratios obtained in the normalization procedure were dependent on a relatively small number of samples, we doubt that their statistical analysis was valid.

offsetting differences in amount of synapse formation and elimination; but there would be a relative increase in size for inactive motor units in the rabbit soleus (present result.) This hypothesis would predict that if the differential activity in the lumbrical had been maintained until the entire muscle was singly innervated, the stimulated motor units would have ended up smaller in size relative to the unstimulated units.

The studies reported by Ribchester and Taxt (1983) and by Ribchester (1988) also involved the lumbrical muscle of the rat. In the earlier study, the muscle nerve was crushed, and a TTX cuff was applied to one of the two nerves that join to supply the lumbrical innervation. They found that active neurons on average innervated twice as many muscle fibers as inactive neurons. In the more recent study, Ribchester (1988) crushed the larger of the two nerves supplying the lumbrical muscle, which led to massive collateral sprouting by motor axons from the remaining nerve. He then compared the extent of reinnervation by inactive axons versus that of normally active regenerating axons in this situation, where the regenerating axons had to compete with existing synapses on active muscle fibers. Once again, the results show that the eventual size of regenerated motor units was considerably smaller for inactive axons than for active axons.

These findings clearly demonstrate a net advantage to active motor neurons in reinnervation of adult rat muscle. In order to reconcile this with our own findings, we can again invoke the fact that the experiments were carried out in different species, different muscles, and at different ages. However, there are several important differences in the experimental paradigms used, and these provide other alternatives that could account for the apparent discrepancies in results. There are several ways in which differential activity could confer a competitive advantage in the reinnervation experiments. We have raised the possibility that inactivity might

reduce the capacity to form new synapses. In addition, inactivity might reduce the rate of axonal regeneration and/or the ability to respond to sprouting influences. Any of these possibilities would put inactive motor neurons at a disadvantage in an adult reinnervation paradigm but would have little or no effect on normal postnatal synapse elimination, where axons are not regenerating and there is little tendency for sprouting to occur even under conditions that induce massive sprouting in the adult (Brown et al., 1981).

Another important consideration is that the regeneration paradigm involves an inherent asymmetry in the occurrence of sprouting signals. Inactive muscle fibers should continuously release a sprouting signal that would occasionally induce collateral sprouting and synaptic takeover by active motor neurons. In contrast, active muscle fibers no longer release overt sprouting signals and thus are more likely to remain stably innervated. Consequently, any muscle innervated by a mixture of active and inactive neurons is inherently in an unstable state; the competition at the dually innervated endplate will result in the removal of one input, but if that leaves the fiber innervated by only an inactive input, the subsequent production of sprouting signal will tend to restore an active input. Thus, there may be a tendency for asymmetric sprouting, involving gradual encroachment by active motor neurons onto inactive muscle fibers, even though the probabilities of survival at a dually innervated endplate could initially be equal or could even favor the inactive synapse. In principle, this asymmetry applies to normal neonatal muscles as well as to reinnervation in the adult, but it is probably much less important in the former, owing to the reduced tendency for sprouting to occur in neonatal muscles.

An *in vitro* system in which more active synapses are preferentially retained during elimination has been described by Magchielse and Meeter (1986). In their system, chick ciliary (parasympathetic) neurons were made to innervate skeletal

muscle fibers polyneuronally, but the multiple inputs were distributed across spatially separated endplates. Stimulation-induced elimination of endplates was subsequently observed. When unstimulated synapses shared innervation of a muscle fiber with stimulated synapses, the unstimulated endplates were preferentially removed. The difference between their result and ours may reflect the difference in motor neuron type or possibly the lack of direct competition between synapses sharing a single endplate. These results suggest that synapse elimination might be regulated differently in different neuromuscular systems (i.e., on smooth muscle versus skeletal muscle).

Combined pre- and postsynaptic partial inactivity. The experiments in which nearly half of the soleus motor axons were inactivated by TTX (TTX major block animals) led to several significant findings. First, inactive motor units were larger than normal for both the fast and slow populations in all 3 muscles examined. This is not surprising given that complete nerve inactivation is known to slow down synapse elimination markedly (see Thompson, 1985, for review). A plausible explanation is that inactive muscle fibers release a sprouting or trophic factor (cf. Brown et al., 1980) that would reduce the natural tendency of neonatal motor neurons to withdraw their terminals.

Unexpectedly, it was found in these experiments that active motor units were significantly larger than normal for the fast population, but less so or not at all for the slow population. How might one account for an effect on active motor units that differs for fast and slow types? One possibility is that inactive muscle fibers might release a diffusible factor that reduces the rate of synapse elimination at neighboring endplates. The differential effect would be explained if for any reason the sprouting factor operated more effectively or at a lower threshold on fast terminals compared to slow terminals. Alternatively, there could conceivably

be separate factors operating independently for fast and slow populations; the differential effect could arise from greater production of the fast-sprouting factor or greater sensitivity to a given concentration. Still another possibility, unrelated to sprouting, is that graded changes in postsynaptic activity might reduce the rate of synapse elimination on fast fibers but not on slow fibers. Although we cannot rule it out, this effect seems unlikely, given that we found no discernible change in the overall rate of synapse elimination in our main experiments involving a less extensive activity block (see above).

Differential versus correlated activity. A "Hebbian" synapse is one in which correlated pre- and postsynaptic activity leads to strengthening of the synapse (Hebb, 1949). Our results indicate that the neonatal neuromuscular junction is not Hebbian. However, there are three conceptually distinct ways in which this non-Hebbian behavior might be mediated. These are separated into two classes: 1) those in which activity levels are important but the correlation between pre- and postsynaptic activity is irrelevant; and 2) those in which correlation is important, but the sign of the effect is opposite to that predicted by Hebb's hypothesis (anti-Hebbian).

Two general mechanisms can be distinguished in the first class. In one, the competitive ability of presynaptic terminals is influenced only by their own level of activity (1a). If it were the level of presynaptic activity *per se* that determined a terminal's competitive ability, the winner would simply tend to be the terminal with the least activity. In the second mechanism, the level of postsynaptic activity influences terminal competence (1b). For this mechanism, presynaptic activity remains important only insofar as it is required to trigger postsynaptic activity. This mechanism differs from #1a in that presynaptic activity at any given terminal has an equal influence on all of the terminals at the same endplate; this influence is

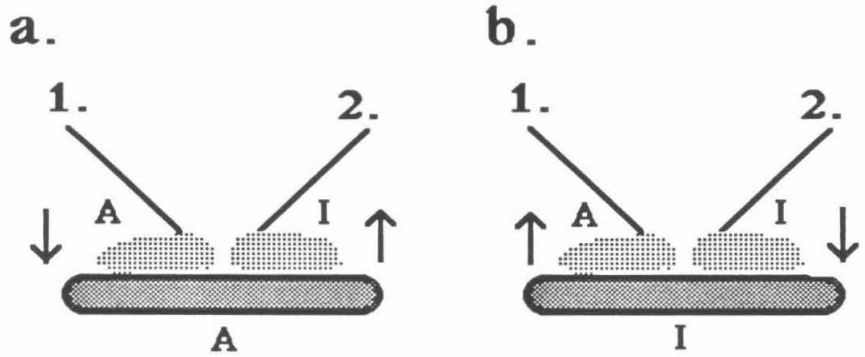
mediated by the activity of their common postsynaptic element. But if this is the case, how can this mechanism confer an advantage to less active motor neurons? One possibility is that reduced postsynaptic activity at a given endplate might result in increased competence for all the terminals of a particular motor neuron innervating that endplate. For example, decreased postsynaptic activity might cause enhanced release of a retrogradely transported trophic factor that enhances the growth capacity of motor neurons (see below and also Chapter 2).

The second, or anti-Hebbian class of mechanisms can be broken into four different cases illustrated in Fig. 10. Figures 10a and 10b each depict a single endplate with presynaptic input from two different motor neurons. The "A"s and "I"s in the figure reflect whether a particular pre- or postsynaptic element is active or inactive at a particular point in time. Figures 10a and 10b will therefore be used to consider whether a given input (1–active or 2–inactive) should be conferred an advantage or disadvantage when the postsynaptic cell is simultaneously either active (Fig 10a) or inactive (Fig 10b). For Fig. 10a, the active presynaptic input should be at a disadvantage because of the concurrent postsynaptic activity (case 2a), while the inactive presynaptic input should receive an advantage (2b). For Fig. 10b, inactivity would be a disadvantage for the presynaptic element because of the paired postsynaptic activity (2c), while activity would be an advantage(2d).

At least one of these cases, an advantage to terminals that are active when the postsynaptic cell is not (2d, terminal 1 of Fig. 10b), is probably not relevant for the specialized case of the neuromuscular junction. Presynaptic activity in the absence of postsynaptic activity cannot occur unless it is assumed that generation of an action potential is the relevent postsynaptic response, in which case only subthreshold inputs would be competent to receive the advantage. Thus, single innervation would never be achieved because inputs would be weakened to

Figure 10. Diagarams illustrating the four possible temporal relations between pre- and postsynaptic activity, with arrows indicating whether the particular correlation between pre- and postsynaptic activity in each case would have a positive (up arrow) or negative effect (down arrow) in an anti-Hebbian system. For the particular point in time represented by the diagrams, elements that are active are indicated by "A" and inactive elements by "I". Thus, in both **a** and **b**, presynaptic terminal 1 is active and presynaptic terminal 2 is inactive. However, the sign of the effect of activity states is opposite in **a** versus **b** because of the opposite signs of the postsynaptic activity. In **a**, correlated pre- and postsynaptic activity confers a disadvantage to terminal 1, but the anticorrelation for terminal 2 – postsynaptic activity in the absence of presynaptic activity – confers an advantage. On the other hand, in **b**, terminal 1 receives a positive influence because it is active while the postsynaptic cell is inactive, and for terminal 2, the correlated inactivity confers a disadvantage.

FIGURE 10



subthreshold status, whereupon they would be strengthened rather than eliminated. On the other hand, such a mechanism might be relevant in systems where multiple innervation persists in adulthood, and simultaneous firing of several presynaptic elements can sum to generate a postsynaptic spike. One such example is particularly noteworthy here, as it points out that such mechanisms may indeed be at work in other systems, and alos shows that the distinctions in Fig. 10 are meaningful. During the formation of ocular dominance columns in the visual system, the rearrangement of connections apparently follows Hebbian rules (see Singer, 1987, for review). Reiter and Stryker (1987) coupled monocular deprivation with infusion of muscimol, an inhibitory agonist. In this situation, the inputs from the normal (more active) eye were at a disadvantage (in contrast to their being at an advantage in the absence of muscimol). The result suggests that in this Hebbian system, anti-correlation is a disadvantage; specifically, presynaptic in the absence of postsynaptic activity is a disadvantage.

Possible cellular signals. The present study has been directed at an understanding of the "rules" of synaptic competition, i.e., how the winners are decided. Ultimately, this approach needs to be integrated with an understanding of the actual cellular signals and events during synapse elimination. A variety of possible signals have been proposed to play an important role during synapse elimination. These include a diffusible trophic factor (Thompson et al., 1979), a stable scaffolding factor in the extracellular matrix (Van Essen, 1982), and calcium-activated protease associated with either the presynaptic or postsynaptic elements (O'Brien et al., 1978; 1984; Connald et al., 1986).

The protease model is the only one for which there is supportive experimental evidence specific to neonatal synapse elimination. Both low calcium and inhibition of protease can slow the rate of synapse elimination (Connald et al., 1986).

Activation of the protease by calcium is hypothesized to cause the disruption and eventual elimination of presynaptic terminals. Since protease has been shown to be present in both muscle fibers (O'Brien et al., 1978) and neurons (Schlaepfer and Hasler, 1979), either source could potentially influence synapse loss. It is interesting to consider that if presynaptic proteases were important, an advantage to the less active terminals would be expected – just as we have observed experimentally. More activity would be associated with greater calcium influx, and hence there would be greater protease activation, leading to degradation of the terminal. Therefore, the lower competitive ability of active synapses in our experiments might reflect an effect of presynaptic protease. This mechanism is a relatively simple (as well as plausible) example that falls into the category of regulation according to level of presynaptic activity *per se*.

It is also possible to construct hypotheses that are anti-Hebbian and consistent with one or more of the 4 variations considered above. For example, postsynaptic activity correlated with presynaptic could be a disadvantage if recently active nerve terminals were more susceptible to postsynaptic activity dependent protease because of the incorporation of membrane material associated with vesicle release. There are numerous other potential mechanisms; however, in the absence of any experimental evidence, they must all be considered speculative.

In the trophic factor model, it has been presumed that activity increases uptake (Thompson, 1985), which at first glance would seem to give an advantage to more active synapses. This need not be the case, however. For instance, the opposite outcome might occur if activity also accelerated the turnover of trophic factor or the amount of the factor needed in order to remain competitive. Another possibility derives from the hypothesis that the production of trophic factor by the muscle fibers might be inversely related to activity (Thompson et al., 1979). Evidence

for a relationship of this sign comes from numerous studies in which the rate of elimination is shown to be proportional to overall activity levels (see Thompson, 1985, for review). Accordingly, neurons with low activity would innervate muscle fibers producing, on average, high levels of trophic factor; neurons with high activity would innervate muscle fibers whose aggregate level of trophic factor production would necessarily be somewhat lower. If the trophic factor were to enhance the survival probabilities of all synapses belonging to a motor neuron, then less active motor neurons would gain an advantage. In order for this mechanism to be effective, however, the influence of the trophic factor would have to be restricted to the terminals (and their sibling terminals) innervating the muscle fiber that produced it. This example falls into the category of mechanisms in which postsynaptic activity is critical.

It is important to note that an advantage to less active terminals is only one observation to be accounted for by any proposed mechanism. Perhaps the most critical additional observations are i) that terminals are in direct competition (as indicated by partial denervation experiments; Brown et al., 1976; Thompson and Jansen, 1977; Betz et al., 1980; Fladby and Jansen, 1987) and ii) endplates become singly innervated in the final state without the appearance of denervated endplates (Brown et al., 1976). Of the mechanisms described above, only the "postsynaptic" mechanism can on its own explain these additional observations. The presynaptic mechanism provides no substrate as the object of competition, nor does it provide a mechanism for the maintainance of the final synapse.

Similarly, the anti-Hebbian possibilities outlined above require additional signals or cellular interactions. If correlated activity were a disadvantage, what would prevent elimination of the final synapse, whose pre- and postsynaptic activity would be completely correlated? As noted above, if there were an advantage

to presynaptic elements that were active in the absence of postsynaptic activity, terminals would weaken until they could not induce a suprathreshold postsynaptic response, whereupon they would be strengthened and reactivated rather than eliminated. If an inactive presynaptic element is strengthened when correlated, postsynaptic activity occurs, this requires the presence of a second terminal to induce strengthening. Thus, one synapse could be strengthened only so long as another elicited postsynaptic responses; once the other synapse was weakened to subthreshold status, it would be preferentially strengthened by the postsynaptic activity elicited by the suprathreshold terminal. Again, it would be difficult to eliminate synapses at all by this mechanism. This is not to say that these mechanisms are not feasible, but that a more complex set of interactions must be invoked to explain the known experimental observations.

On the other hand, in the postsynaptic mechanism described above, one only need suppose that the aforementioned trophic factor is the object of competition to account for the system's known behavior. The factor need not be taken up by the presynaptic terminals in a manner dependent on their activity, except insofar as presynaptic activity determines the level of postsynaptic activity and subsequently the level of factor. Reduction of the number of terminals per endplate would be assured by the competitive interactions, but loss of the final synapse would be avoided by the absence of competition in the singly innervated state and by the trophic influence from muscle.

Functional significance. According to the size principle (Henneman and Olson, 1965), the recruitment threshold of a motor neuron is directly related to its motor unit size – smaller motor units are recruited first and progressively larger motor units are recruited as excitation of the motor pool increases. The presumed advantage of this configuration is that force generated by a large muscle can be finely

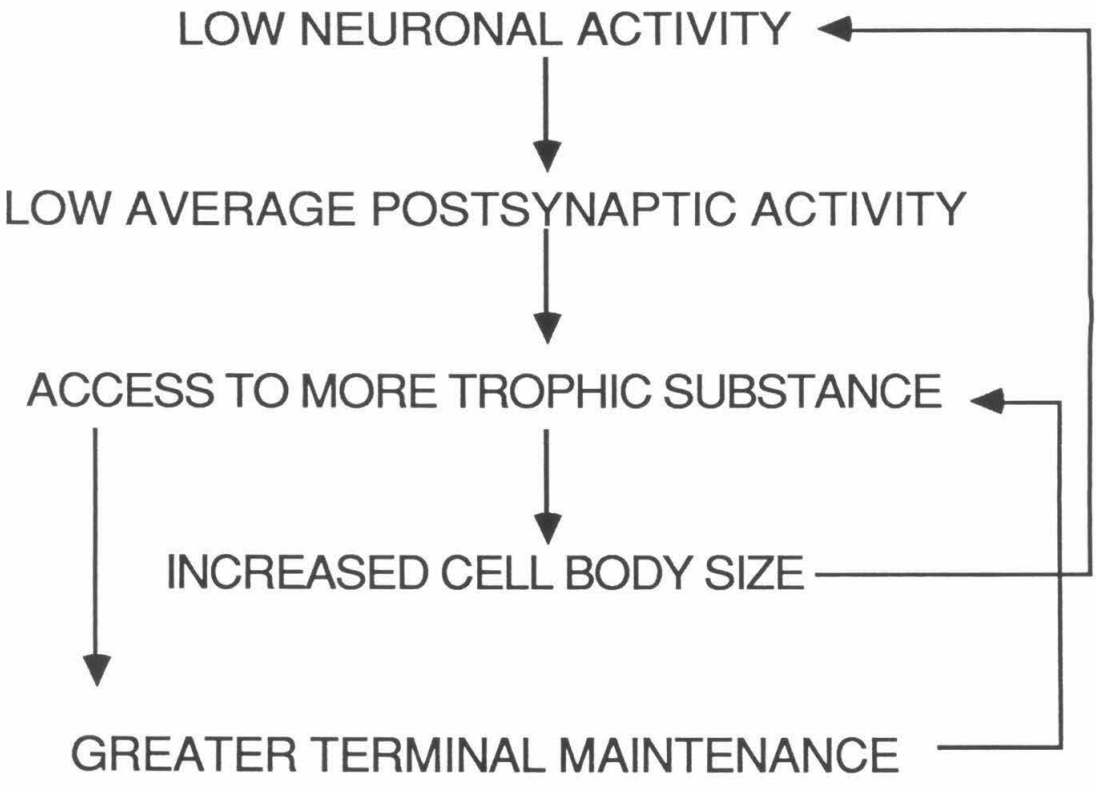
graded when small movements are needed, while maximal force can be generated with a moderate number of motor neurons. How these relationships are established during development is not known. It is interesting to speculate that the relationship between recruitment threshold and motor unit size might come about as the result of preferential retention of less active inputs during synapse elimination. If a motor neuron's level of activity at birth is closely related to its adult recruitment threshold, such a role for activity in the regulation of neuromuscular synapse elimination seems quite likely.

In this regard, it is once again of interest to consider the proposed postsynaptic *trophic* mechanism. If the trophic substance could influence the size of a motor neuron's cell body, as well as the stability of its terminals, a positive feedback loop could be generated (Fig. 11). Less active motor neurons (those with relatively large cell bodies; Henneman and Olson, 1965) would innervate muscle fibers that are less active on average and would therefore have greater access to the trophic substance (as already discussed above). The greater trophic influence would promote both greater terminal competence and an increase in cell body size, each of which could have their own positive feedback effect. The increased cell body size would lower the input resistance and could thus raise the threshold for activation. This could further decrease activity, resulting again in greater production of trophic substance; and the greater survival of synapses would maintain access to the trophic substance at more sites. According to this model, the burden for development of correlated recruitment threshold and motor unit size is not solely on activity dependence. A motor unit's recruitment threshold and size would both be altered until the two values converged at appropriate points along a continuum.

An advantage to more active inputs and/or strengthening of connections with correlated pre- and postsynaptic activity have been demonstrated in both the

Figure 11. Diagram of the positive feedback loops in the proposed mechamism for development of motor unit recruitment threshold to size matching. Both motor neuron cell body size and terminal competence would be dependent on a trophic substance produced by muscle fibers according to their activity level. Less active motor units would have access to more trophic substance because of the lower average activity levels of the endplates they innervate. This would result in cell body growth and further decrease in activity. The greater terminal competence would result in maintenance of more terminals which would also allow for continued access to trophic substance at more endplates.

FIGURE 11


LOW NEURONAL ACTIVITY
LOW AVERAGE POSTSYNAPTIC ACTIVITY
ACCESS TO MORE TROPHIC SUBSTANCE
INCREASED CELL BODY SIZE
GREATER TERMINAL MAINTENANCE

development of the visual system and in long-term potentiation in the hippocampus (Singer, 1987; Kelso et al, 1986). In the case of the hippocampus, it is clear that the sign of the effect is quite appropriate to the function of the system. Learning is, in fact, the function for which Hebb formulated his hypothesis (Hebb, 1949). In the visual system (see Singer, 1987 for review), early segregation of inputs from left and right eyes is apparently crucial to generation of more complex response properties. A Hebbian system is apparently ideal for generating this segregation, since the activity within an eye is more correlated than that between eyes. In a similar system, ocular dominance stripes are produced in three-eyed frogs in which inputs from two eyes normally do not overlap but topographic projections do develop (Constantine-Paton and Law, 1978); in addition, the segregation of stripes from initially overlapping projections is dependent on activity (Reh and Constantine-Paton, 1985). Here it appears that a mechanism normally responsible for subserving one function, refinement of topography, can also cause a more dramatic segregation, given a higher degree of non-correlation.

A competitive advantage to less active synapses has not, to our knowledge, been described in any system previously. However, the importance of Hebbian mechanisms in some systems and the elegance of the hypothesis certainly do not mandate the presence of such a mechanism in all activity-regulated neural systems. Hopfield et al. (1983) have demonstrated that in simulated neural networks, synaptic weakening during correlated pre- and postsynaptic activity is useful for the removal of spurious memory states. Also, E. Frank (personal communication) has hypothesized that if activity played a role in specifying connections between muscle afferents and motor neurons, the system might also be regulated in an anti-Hebbian manner, since the appropriate afferents fire when the muscle is stretched, *not* when the motor neurons are firing and evoking contraction. However, this

interpretation is not straightforward; for example, the sign might be opposite for specifying connections to γ-motor neurons, and very little is known about the actual temporal relations between motor and afferent activity *in vivo*. We feel that the neuromuscular system may not be the only one in which activity is a disadvantage for retention of connections; rather, the sign of an activity influence, if any, should relate to the function of the system in question.

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