THE FORMATION AND LOSS OF SUPERNUMERARY SYNAPSES IN MAMMALIAN SKELETAL MUSCLE

Thesis by

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Abstract

Physiological and anatomical techniques have been employed to investigate the elimination of polyneuronal innervation in skeletal muscles of the neonatal rabbit. In an initial study involving intracellular recording and *in vitro* tension measurements, it was discovered that different muscles in the rabbit differ with respect to the extent of multiple innervation and the time course of synapse elimination, in a way that suggests a body-wide gradient of neuromuscular development. Counts were then made of both α-motor neurons (using retrograde transport of horseradish peroxidase) and muscle fibers for each of three rabbit muscles, to see whether the differences between muscles in the degree of multiple innervation and/or the rate of synapse elimination were related to the differences in the motor neuron:muscle fiber ratio (the innervation ratio). The results suggest that individual motor neurons from muscles with a 10-fold range of innervation ratios tend initially to establish the same number of synapses, which results in different degrees of multiple innervation among muscles. The rate of synapse elimination in a muscle, however, does not appear to depend on the innervation ratio.

An electron microscopic study on the morphological correlates of synapse elimination yielded three main findings: one, synapse elimination appears to involve retraction rather than degeneration of synapses; two, myelination of the pre-terminal axons seems to proceed independently of the loss of synapses; and three, synapses in neonatal muscle can exhibit unusual morphology indicative of their rapidly changing state.

A related study was carried out on adult rat muscle. Physiological and anatomical evidence was found that a foreign nerve transplanted

onto a muscle with intact innervation is capable of forming and maintaining connections, if its axons grow to the sites of original nerve synapses.

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General Introduction

One of the more difficult and intriguing general problems in biology is to understand the complex process by which an organism transmutes from egg to adult. The development of the nervous system is a particularly interesting aspect of this problem, to sentient beings like ourselves. Moreoever, the functioning of the mature nervous system is likely to depend on a dynamic state in which events quite like those of development may routinely take place. Thus many areas of neural development acquire a kind of dual fascination for biologists. In particular, the formation, maintenance, and elimination of synapses make up, collectively, a process which not only plays a crucial role during development, but is suspected to be of major importance for the successful operation of the adult nervous system. It is well documented that the mature nervous system is capable of forming new synapses in situations where trauma or disease lead to the loss of normal connections (Cotman and Lynch, 1976). There is some evidence to suggest that loss and renewal of synapses is an ongoing process in both the peripheral (Barker and Ip, 1966) and central nervous systems (Sotelo and Palay, 1971), though this is disputed (Tuffery, 1971). One can imagine, furthermore, that complex phenomena like memory and learning involve the formation of appropriate connections in the brain and/or the removal of inappropriate connections, by mechanisms analogous to those which ensure the establishment of correct synapses during development.

This process of formation and removal of neuronal connections is the object of the studies detailed in this thesis. Like many experiments

dealing with dynamic interactions at synapses, these are designed to enable a formulation of the rules governing synapse formation and elimination, rather than the precise molecular mechanism underlying such rules. I believe, however, in common with many developmental biologists, that this kind of phenomenological research is a prerequisite for the more fundamental understanding which is an eventual goal.

In 1970, Redfern discovered that, during early postnatal mammalian development, muscle fibers receive multiple inputs, which could be demonstrated by the presence of endplate potentials with several components (Redfern, 1970). Within a few weeks after birth, all but one component disappear, resulting in the situation seen in adult muscle. Redfern suggested that these phenomena resulted from an initial over-production of neuromuscular synapses, followed by a period of synapse elimination leading to each muscle fiber being singly innervated. Since that time, several studies have confirmed his suggestion and attempted to elucidate the mechanisms by which this synapse loss occurs, as well as the advantage conveyed to the animal by such a process (Bennett and Pettigrew, 1974; Riley, 1976; Brown et al., 1976; Thompson, Kuffler and Jansen, 1979). In other investigations it was discovered that developmental synapse elimination is a rather general phenomenon in mammals, occurring in such diverse regions as autonomic ganglia, the cerebellum, and the spinal cord in addition to muscle (Conradi and Ronnevi, 1977; Crepel et al., 1976; Lichtman, 1977). While these findings provide further incentive to investigators wishing to draw general conclusions, the advantages of working with large cells and readily accessible peripheral tissues make the neuromuscular junction the most advantageous for detailed analysis. There are several questions which suggest

themselves immediately as the foci for such an analysis. What factors control the extent of the initial multiple innervation? On what basis is the decision made to eliminate a given synapse; i.e., pre-programmed instructions, competitive interactions among nerve terminals, matching of activity between nerve and muscle, etc.? What cells are involved in the elimination process? What is the physical nature of the synapse loss--degeneration or retraction? These questions form the rationale for the experiments described in this paper.

The first principle to be deduced for neuromuscular synapse elimination is that it is unlikely to be the result of decisions made solely by the nerve or muscle, but instead that interactions between nerve and muscle are probably important in the selection of synapses for survival (Brown et al., 1976). Other experiments suggested that the process involves both a tendency for motor neurons to reduce their peripheral fields at a certain developmental age, and a competition between axons converging on a single endplate site (Brown et al., 1976; Thompson and Jansen, 1977). Such a competition may take place on the basis of differences among terminals in the amount or pattern of electrical activity (Benoit and Changeux, 1975, 1978; Thompson et al., 1979). Attempts to determine the exact means by which motor axons lose functional synaptic contacts led to two hypotheses: that terminals to be eliminated were "sloughed off" by the parent axon and degenerated in place, or that these terminals were simply retracted into the parent axon (Korneliussen and Jansen, 1976; Rosenthal and Taraskevitch, 1977; Riley, 1977), in a process analogous to that observed in axonal growth cones in vitro (Speidel, 1933; Bray, 1970).

It was with this general background that the experiments presented here were undertaken. The thesis is in four parts. Chapter I describes the time course of synapse elimination in several muscles of the neonatal rabbit; this serves also as an introduction to a system in which it is hoped that experimental manipulations designed to test ideas about elimination can be readily achieved. In Chapter II, the differences among muscles in initial degree of multiple innervation and peak rate of synapse elimination are further explored. In particular, these experiments test the idea that differences in these parameters are related to variations among muscles in the ratio of motor neurons to muscle fibers. The conclusions are that the degree of multiple innervation in a muscle depends on this ratio, in a way which suggests an intrinsic limit to the size of a neuron's peripheral field, but that rates of elimination seem to be independently controlled. Chapter III is an electron microscopic study of developing neuromuscular innervation, which addresses two main issues. One, is myelination by Schwann cells involved in the selection of synapses for survival; and two, does synapse elimination occur by degeneration or retraction of terminals? The results strongly favor retraction as the predominant mechanism, but provide no evidence for the involvement of Schwann cells in this decision. In addition, observations are reported concerning the dynamic nature of nerve-muscle interactions at these early stages. These three chapters together allow partial answers to the questions posed earlier in the introduction, and pave the way for further exploration in the same system. Chapter IV is a separate study dealing with synapse formation and maintenance in adult animals. Using the adult rat as an experimental model, these experiments show that the lability necessary for supernumerary synapse formation and

subsequent elimination can be expressed in adult muscle, thus encouraging the idea that the kind of processes studied during development can also occur in the mature nervous system.

Many of these experiments were done in collaboration with David C. Van Essen, and John H. R. Maunsell was instrumental in the work described in Chapter II.

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Chapter I

Regional Differences in the Timing of Synapse Elimination in Skeletal Muscles of the Neonatal Rabbit[1]

Mammalian skeletal muscles characteristically undergo dramatic changes in their pattern of innervation around the time of birth. The most prominent events are an initial overproduction of synapses, resulting in the polyneuronal innervation of individual muscle fibers, and a subsequent period of synapse elimination, such that mature fibers receive an input from one and only one motor axon (Redfern, 1970; Bagust, Lewis, and Westerman, 1973; Bennett and Pettigrew, 1974; Brown, Jansen, and Van Essen, 1976). The process leading to the removal of "extra" neuromuscular synapses is of general interest, since similar phenomena occur during the maturation of other regions of the nervous system, including autonomic ganglia (Lichtman, 1977) and portions of the central nervous system (Crepel, Mariani, and Delhaye-Bouchaud, 1976; Conradi and Ronnevi, 1977; see also Rakic, 1977; Hubel, Wiesel, and LeVay, 1977; LeVay, Stryker, and Shatz, 1978). The present study is principally concerned with the timing of synapse elimination in various skeletal muscles of the rabbit. We initially chose this species because it is larger than the rat, the mammal whose neuromuscular development has been most extensively studied, and so is more amenable to certain experimental manipulations designed to explore the mechanism of synapse elimination. A striking feature of

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the process of synapse elimination in the rabbit is that its time course varies considerably in muscles from different parts of the body, something not found in studies on the rat. Our results suggest that there are regional differences of up to a week in the time of onset of rapid loss of synapses, which may be related to a rostro-caudal developmental gradient. In addition, there are differences in the maximal rate of loss of synapses, and possibly also in the peak level of polyneuronal innervation, but variations in these characteristics are not clearly related to body position.

Methods

Experiments were carried out on skeletal muscles from the hindlimb, forelimb, neck, and trunk in late fetal and early postnatal New Zealand White rabbits (Oryctolagus cuniculus). For electrophysiological recording a muscle with its motor nerve was isolated and pinned out in a small glass-bottomed plexiglass chamber. The chamber was perfused at room temperature (23°C) with a well-oxygenated Ringer's solution containing (in mM): NaCl; 150; KCl, 5; $CaCl_2$, 5; $MgCl_2$, 1; D-glucose, 16.5; HEPES buffer (pH 7.4), 5. Most experiments involved intracellular recordings of endplate potentials (e.p.p.'s) using conventional glass microelectrodes beveled to resistances of 30-50 MΩ. D-tubocurarine (1-3 μg/ml) was added to the bath to block nerve-evoked muscle contractions. E.p.p.'s were evoked by stimuli of graded strength applied to the motor nerve through a close-fitting glass suction electrode.

In each muscle the incidence of multiple innervation was assessed by sampling at least ten and usually twenty muscle fibers. It would have been useful in our analysis to determine the precise number of synaptic

inputs to each muscle fiber in the sample. In practice, however, such information was not routinely obtainable for the rabbit muscles we studied. This was principally because large fluctuations (often several-fold) in the amplitude of individual e.p.p. components made it difficult to distinguish reliably between, say, triple and quadruple innervation of a given fiber. We therefore chose simply to score all fibers according to whether or not they were multiply innervated. Consequently, with this method we were unable to detect the early stages of synapse loss for those muscles which initially had many fibers with three or more inputs.

In some experiments, contractile tensions were measured using a stiff piezoelectric bimorph element as an isometric tension transducer. One muscle tendon was pinned to the bottom of the chamber, and the other tendon was attached by surgical thread to the transducer; the output of the transducer was fed into a high-impedance D.C. pre-amplifier. For measurements of contraction speeds the motor nerve was stimulated near the muscle. In other experiments involving the measurement of motor unit tensions, the nerve was dissected back to the ventral roots, and individual ventral root filaments were stimulated in succession. The maximal muscle contractile tension was determined by stimulating the muscle directly with bipolar Ag-AgCl electrodes. The degree of polyneuronal innervation was assessed by determining the tension overlap, i.e., the sum of the individual tension measurements relative to the maximal directly elicited tension. Before summing, though, the individual tension measurements were corrected for tension overlap among different motor units within a filament by a relationship derived from Poisson analysis. Specifically, t', the corrected tension value, was calculated as $t' = T_m \ln (1 - t/T_m)^{-1}$,

where T_m is the maximal direct tension and t is the tension measured from stimulating a filament.[2]

For histological analysis, nerves and muscles were fixed in 2% glutaraldehyde in 0.12 M cacodylate buffer. Semi-thin (0.5-1.0 μm) sections of Epon-embedded tissue were cut on glass knives and stained with toluidine blue. Nerve and muscle fiber counts were made from photomicrographs of these sections. In some experiments, endplates were stained for cholinesterase (Buckley and Heaton, 1968); following formaldehyde fixation, single muscle fibers or bundles containing 2 or 3 fibers were teased out and examined by light microscopy.

Results

An initial series of experiments on the soleus muscle was carried out to determine whether its pattern of innervation in newborn rabbits is similar to that which has been found in other neonatal mammals. We found that all soleus muscle fibers in the rabbit were multiply innervated at birth, as evidenced by the presence after partial curarization of two or more discrete components of the e.p.p. that were separable by gradation of the stimulus delivered to the nerve. We believe that these physiological observations reflect convergent multiple innervation of a single endplate site per muscle fiber, since the rise times of all components

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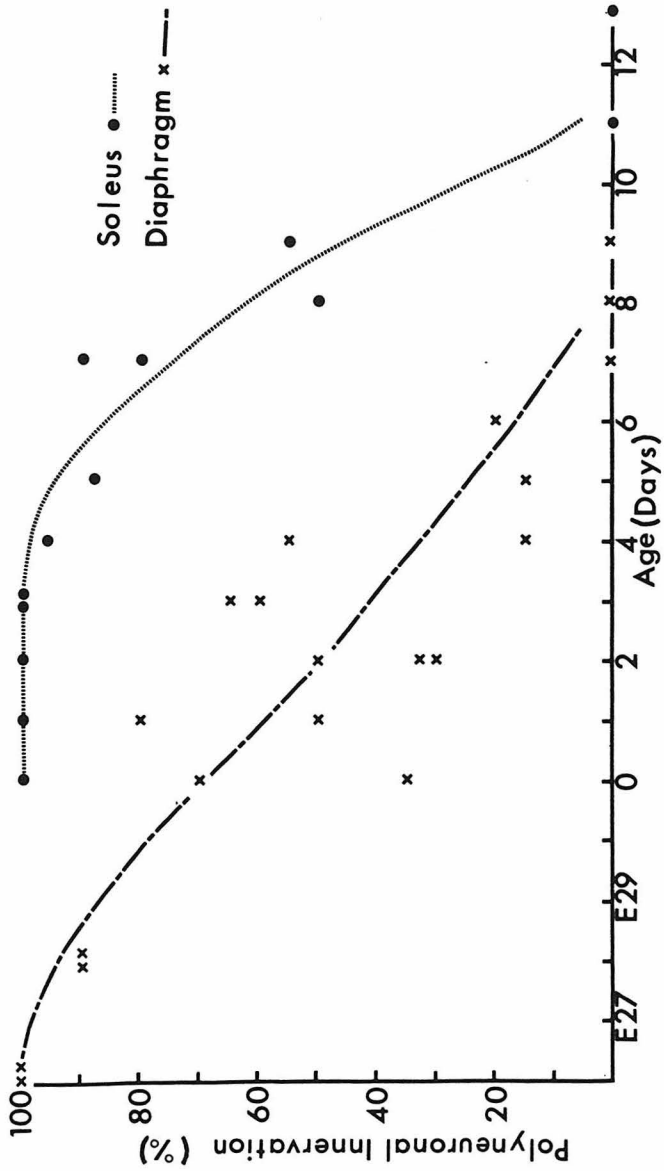
[2] If a filament makes S synapses in a muscle with M fibers, then $t'/T_m = S/M$. If all synapses have the same probability $1/M$ of innervating any given fiber, then from Poisson's theorem, the probability P_o of any fiber not being innervated via the filament is $P_o = \exp(-S/M) = \exp(-t'/T_m)$. Since $P_o = 1 - t/T_m$, it follows that $t' = T_m \ln (1 - t/T_m)^{-1}$. The analysis is valid if synapses from a filament are distributed randomly within the muscle and if motor units are sufficiently small that the possibility of multiple innervation by different branches of the same parent axon can be disregarded.

of the e.p.p. in any one fiber were always indistinguishable; moreover, after acetylcholinesterase staining only one motor endplate was seen on each isolated muscle fiber. Not all of the synaptic inputs to any one fiber are identical, however. In all of the rabbit muscles we examined, as in rat muscle (Redfern, 1970; Rosenthal and Taraskevich, 1977), there were often marked differences between e.p.p. components with respect to amplitude (up to a factor of ten) or latency following nerve stimulation (up to a factor of two).

The disappearance of multiple innervation apparently does not result from the division of muscle fibers during maturation, since there was no major increase in the number of fibers in the soleus muscle after birth (11,042 at day 2; 11,508 at day 16). Nor was there any indication of death of motor neurons after birth, since no loss of myelinated axons from the soleus nerve was detected over the same time period (226 axons at day 1, 237 at day 2, 242 at day 6, 233 at day 16). Thus, the loss of multiple innervation evidently is linked to the retraction of synapses by individual motor neurons, as is the case in rats (Redfern, 1970; Brown et al., 1976) and kittens (Bagust et al., 1973; Riley, 1976).

The time-course of disappearance of multiple innervation differs markedly in various muscles of the rabbit. We first noted this in comparing the soleus and diaphragm muscles (Fig. 1). In the diaphragm, the process of synapse elimination has clearly begun several days before birth and is completed within the first postnatal week. In the soleus muscle the process occupies much of the second postnatal week and involves a more rapid rate of disappearance of multiple innervation than in the diaphragm. However, we were unable to establish the time of onset of synapse elimination from these results, since the loss of synapses from

Fig. 1. The time-course of synapse elimination in the rabbit soleus and diaphragm muscles. In this and subsequent figures, each point represents the percentage of multiply innervated fibers in a single muscle. At least ten and usually twenty or more fibers were sampled in each muscle. Except where noted, the curves were drawn by eye to fit the observations. (E = embryonic day).


Soleus
Diaphragm
Polyneuronal Innervation (%)
100
80
60
40
20
E27
E29
0
2
4
6
8
10
12
Age (Days)

fibers with three or more inputs is not assayed by the procedure used (see Methods). In order to determine when synapse elimination begins in the soleus muscle, we assessed the degree of multiple innervation by a different procedure, involving the summation of tension generated by individual motor units or small groups of motor units. In each muscle, seven to twelve ventral root filaments, each containing from one to ten soleus motor axons, were stimulated individually, and the resulting muscle twitch tensions were measured. The tension overlap, i.e., the excess of the sum of these tension measurements over the maximal tension elicited by direct stimulation, gives an approximate measure of the degree of polyneuronal innervation. The values of the summed tensions, after correcting for tension overlap within a filament (see Methods) were 295%, 297%, and 281%, respectively, for muscles examined at one-half, one, and four days postnatal. (The uncorrected estimates were 241%, 248%, and 251%.) Conversion of these values into estimates of the mean number of synapses per muscle fiber involves several assumptions which may be of limited validity (cf. Brown et al., 1976); nevertheless, the results suggest that there is an average of about three synapses per soleus muscle fiber at birth, and that this number does not decline substantially for several days. Thus, rapid synapse elimination in the soleus muscle evidently begins around postnatal day four, in contrast to the prenatal onset in the diaphragm. We cannot, however, rule out the possibility that synapse elimination actually begins earlier in the soleus, but is masked by the continued formation of synapses. This difference between muscles in the time course of synapse elimination contrasts with the situation in rats, for which the loss of synapses in the soleus and diaphragm occurs approximately in synchrony (Bennett and

Pettigrew, 1974; Brown et al., 1976; Rosenthal and Taraskevich, 1977).

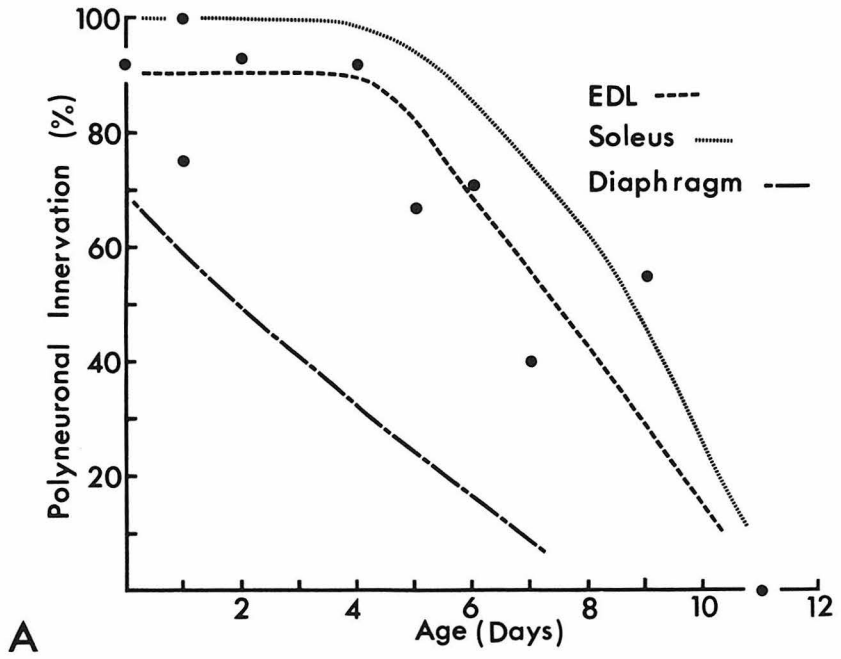
Two characteristics of skeletal muscles that might be related to these differences in synaptic maturation are the location of the muscle in the body and the speed of contraction of the muscle fibers. We therefore examined synapse elimination in four additional muscles, differing in contraction speed and situated in the hindlimb (extensor digitorum longus or EDL), forelimb (pronator teres, anconeus quartus), and neck (levator scapulae major or LSM) (Bensley, 1946).

The results for the EDL are shown in Fig. 2A along with curves for the soleus and diaphragm for comparison. A significant number of singly innervated fibers was found in most EDL muscles examined within the first few days after birth. There was no detectable change in the percentage of fibers receiving multiple inputs before postnatal day four, suggesting a postnatal plateau in multiple innervation and a late onset of rapid synapse elimination like that in the soleus. Strictly speaking, though, this evidence does not rule out the possibility that during the first few postnatal days the EDL contains a fixed population of singly innervated fibers (possibly ones that never were multiply innervated) and a separate population of multiply innervated fibers which undergo a significant degree of synapse loss, but which start with a high number of synapses per fiber.

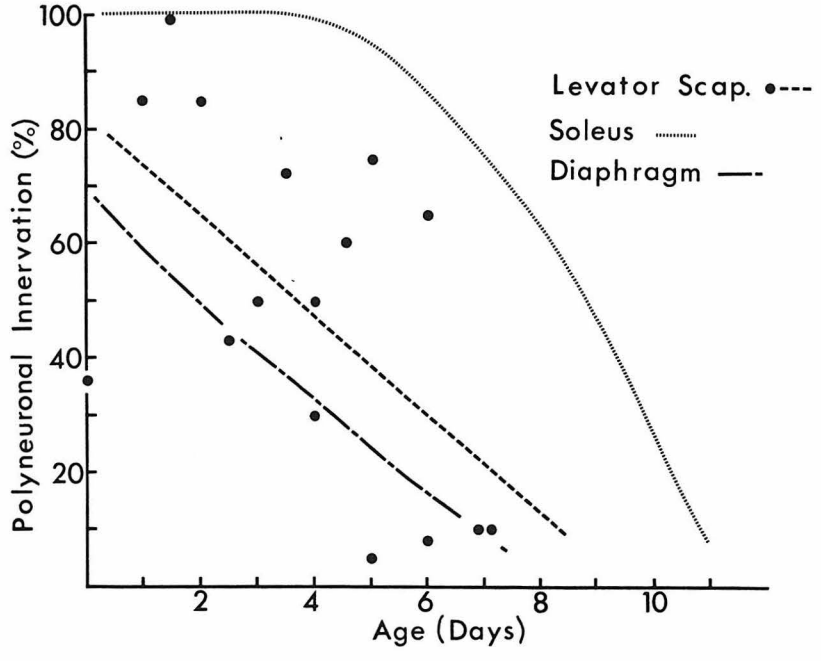
In the LSM, a muscle from the neck, synapse elimination is detectable within a few days after birth (Fig. 2B). As in the diaphragm, there is considerable scatter in the incidence of multiple innervation in different muscles of similar ages, but the slope of the least squares regression line through these points is similar to that for the diaphragm.

The indication of an early onset of synapse elimination in two

Fig. 2. A. Synapse elimination in the extensor digitorum longus (EDL). The curves for the soleus and diaphragm from Fig. 1 have been included for comparison. Note the similarity in time course of synapse elimination between the two hindlimb muscles. B. Synapse elimination in the levator scapulae major (LSM). Soleus and diaphragm curves are shown as in A. The curve for the LSM is a least-squares fit to the data. The onset of elimination is earlier in this neck muscle than in the soleus or EDL.


100
80
60
40
20
Polyneuronal Innervation (%)
EDL ----
Soleus
Diaphragm —
2
4
6
8
10
12
Age (Days)

A

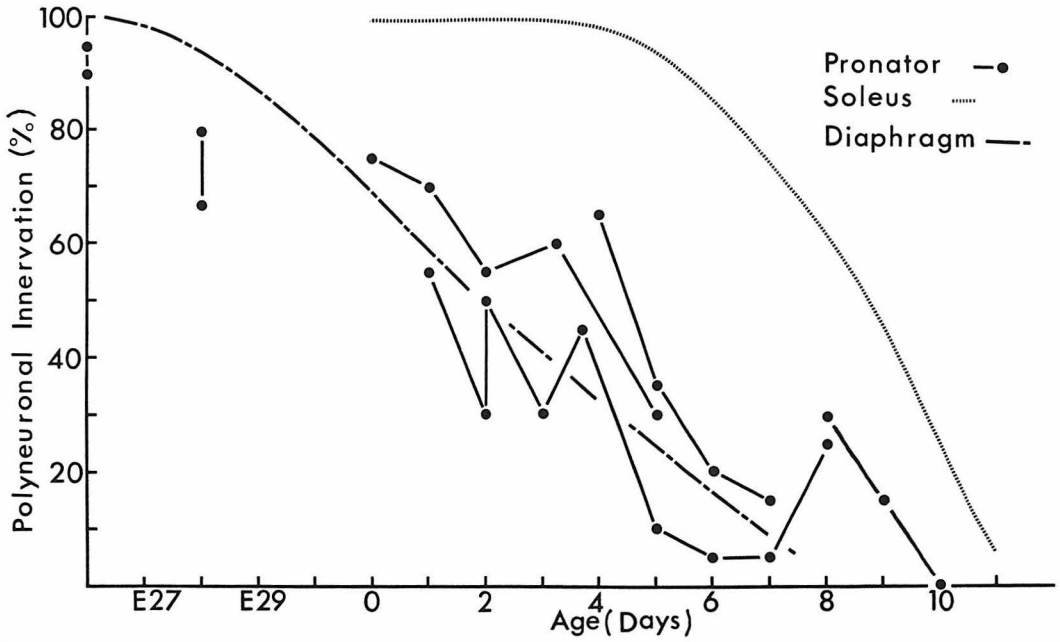

100
80
60
40
20
Polyneuronal Innervation (%)
Levator Scap. •---
Soleus
Diaphragm —
2
4
6
8
10
Age (Days)

B

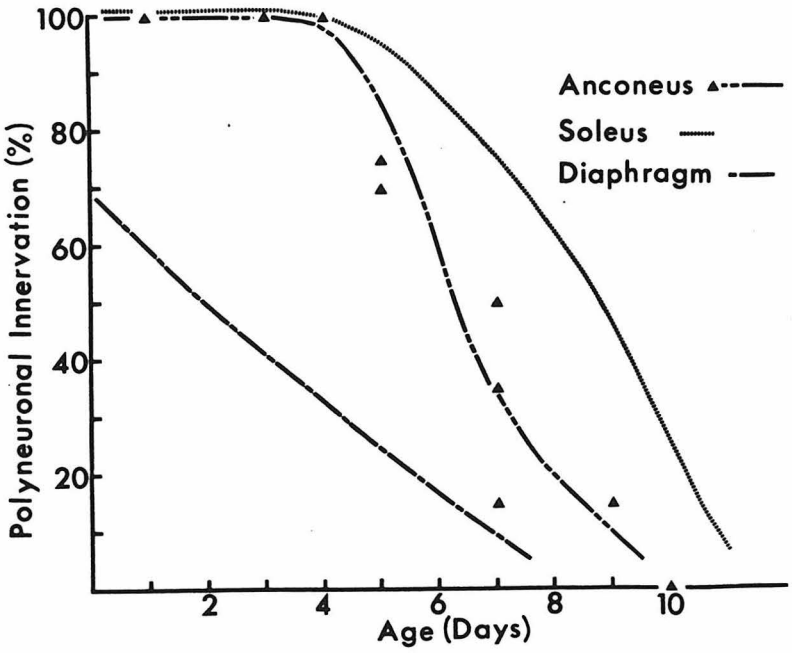
muscles along the body axis and a late onset in two hindlimb muscles suggests a rostro-caudal and/or proximo-distal gradient in the timing of this process. To distinguish between these alternatives, we examined two forelimb muscles, the pronator teres and the anconeus quartus. In the pronator teres, a distal forelimb muscle, synapse elimination begins several days before birth, and continues through the first postnatal week (Fig. 3A). This result suggests that muscle maturation is more closely related to rostro-caudal than to proximo-distal position in the body. In Fig. 3A, points for animals from the same litter are connected by solid lines, and it is apparent that there is less variability within individual litters than in the population as a whole. In several litters there was a suggestion of an enhanced decline in the level of multiple innervation between days 3 and 5, i.e., about the time that rapid synapse loss begins in the soleus and EDL. However, we do not regard this as convincing evidence that the rate of synapse elimination can actually increase once the process has begun.

Fig. 3B illustrates the time-course of synapse elimination in the anconeus quartus, an extensor muscle from the proximal forelimb. Unlike those in the pronator teres, all fibers in the anconeus quartus were found to be multiply innervated when examined during the first few days after birth. The rate of synapse loss is very high in the anconeus quartus, however, and the elimination process is completed at essentially the same time as in the pronator teres. It may be that the number of synapses per muscle fiber is initially very high in the anconeus quartus and, accordingly, that synapse elimination begins well before postnatal day four. On the other hand, it is entirely possible that in this muscle the process indeed does not begin until several days after birth, in which

Fig. 3. Synapse elimination in two forelimb muscles. In both graphs (A and B) the soleus and diaphragm curves are shown as in Fig. 2.
A. Pronator teres. In this graph, no single line has been drawn through the data points. Instead, points for muscles from animals of the same litter are connected by solid lines. There is less variability among animals from a single litter than for the group as a whole. The time course of elimination is similar in the pronator teres and diaphragm.
B. Anconeus quartus. Note the rapid loss of synapses after day four.


100
80
60
40
20
Polyneuronal Innervation (%)
Pronator
Soleus
Diaphragm
E27
E29
0
2
4
6
8
10
Age (Days)

A


100
80
60
40
20
Polyneuronal Innervation (%)
Anconeus
Soleus
Diaphragm
2
4
6
8
10
Age (Days)

B

case one would have to invoke additional factors besides rostro-caudal body position in accounting for the initiation of synapse elimination.

The results for all six muscles are summarized in Fig. 4. In addition to the prominent differences in the time of onset of synapse elimination, there are marked differences among muscles in the peak rate of disappearance of multiple innervation. The slope of each curve presumably gives a reasonable measure of the actual rate of loss of synapses per muscle fiber, at least during the later stages of the process when very few fibers have three or more inputs (Bennett and Pettigrew, 1974; Rosenthal and Taraskevich, 1977). The largest difference in slopes (a factor of about three) is between the two forelimb muscles, anconeus quartus and pronator teres, so there must be other influences besides body position on the rate of synapse elimination. There also appear to be moderate differences in the maximal level of polyneuronal innervation in various muscles, although the difference between the two muscles best characterized in this regard, the soleus and EDL, may be no more than 20-30%. In the rat, estimates of the peak level of multiple innervation in the soleus (Brown et al., 1976) and diaphragm (Bennett and Pettigrew, 1979) muscles differ by nearly a factor of two. It is uncertain whether this difference is genuine, however, since the estimates were based on different methods for the two muscles.

The possibility that certain aspects of synaptic maturation might be related to the contractile properties of muscles was tested by measuring the time-to-peak of twitch contractions in four muscles from mature rabbits. The results are shown in Table I, with the muscles arranged in order from slowest to fastest. For comparison, the third column indicates the approximate time of onset of synapse elimination,

Fig. 4. The time-course of synapse elimination in six skeletal muscles of the rabbit. The curves for the pronator teres and LSM are least-squares fits to the data. Note the differences among muscles in time of onset of synapse elimination and rate of loss of synapses.

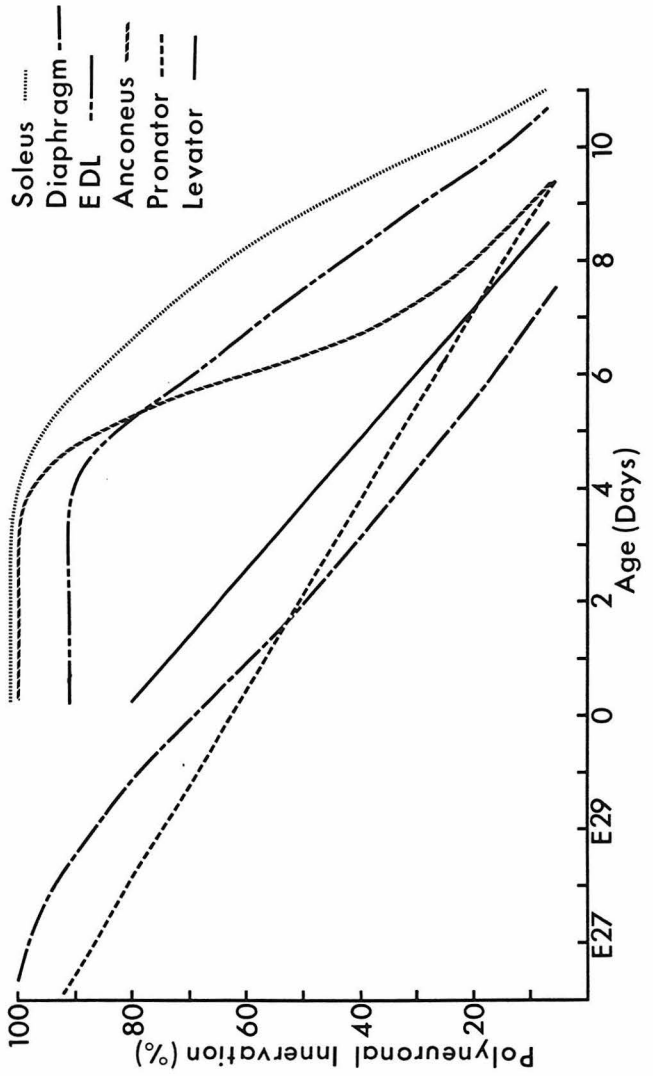

Soleus
Diaphragm
EDL
Anconeus
Pronator
Levator
Polyneuronal Innervation (%)
100
80
60
40
20
E27
E29
0
2
4
6
8
10
Age (Days)

Table I

Contraction speeds and indices of synaptic maturation in rabbit muscles

Muscle	Twitch time-to-peak (msec)	Onset of synapse loss (Embryonic (E) or Postnatal (P) age in days)	Peak rate of synapse elimination (synapses/fiber/day)
Soleus	55	$\approx$ P4	0.14
Diaphragm	35	$\leq$ E26	0.08
Pronator teres	29	$\leq$ E26	0.06
EDL	21	$\approx$ P4	0.12

and the fourth column shows the peak rate of synapse loss, expressed as synapses per muscle fiber per day. As expected from histochemical data from the rabbit and physiological results from other species (Bárány, Bárány, Reckard, and Volpe, 1965; Padykula and Gauthier, 1970; Close, 1972), the soleus and EDL are slow-twitch and fast-twitch muscles respectively, and the diaphragm and pronator teres have intermediate contractile properties. There is no clear correlation, however, between contraction speed and either of the maturation parameters shown. In fact, the muscles with the largest difference in contractile properties (the soleus and EDL) are remarkably similar in time course of synapse elimination.

Discussion

The significance of our observations on the timing of the synapse elimination process can be discussed most profitably in relation to other temporal patterns associated with neuromuscular development in mammals. Progressive development along both rostro-caudal and proximo-distal axes has been demonstrated for several developmental phases, including the initial differentiation of motor neurons (Angulo, 1940; Romanes, 1941; Barron, 1943; Harris-Flanagan, 1969) and muscles (Lewis, 1901; Bardeen and Lewis, 1901; Boethius, 1969), the formation of functional neuromuscular connections, as assessed by the onset of spontaneous embryonic movements (Windle and Griffin, 1931; Barron, 1941), and the subsequent establishment of reflex-activated movements (Barcroft and Barron, 1939; Barron, 1941). The largest temporal differences, between the extremes of each axis, range from less than a day to more than a week, depending on both the gestation period of the species and the specific

developmental process under consideration. The most extensive observations have been made on the onset of embryonic motility, which occurs about the time that the first functional neuromuscular synapses are formed (Strauss and Weddell, 1940; Bennett and Pettigrew, 1974). In mammals such as the cat and sheep, with gestation times of more than a month, the motile period begins relatively early during development, and the temporal differences along rostro-caudal and proximo-distal axes are on the order of several days to a week or more (Windle and Griffin, 1931; Barcroft and Barron, 1939). In the rabbit, spontaneous movements begin midway through gestation (day 15 or 16 out of 31 days; Pankratz, 1931), but information on regional differences in the development of motility is not available.

In view of the clear evidence for a dual set of gradients (rostro-caudal and proximo-distal) in certain aspects of mammalian neuromuscular development, it is interesting that our results on the onset of synapse elimination in the rabbit suggest a rostro-caudal gradient but no obvious proximo-distal gradient. It would not be surprising if studies on other muscles, particularly in more distal parts of the limb, revealed some proximo-distal differences, but any such differences apparently are less prominent than those along the rostro-caudal axis.

Comparison of neuromuscular development in the rat and rabbit suggests significant species differences both in the rate of synapse elimination and in the magnitude of regional differences in its time course. The peak rate of synapse loss is two- to three-fold higher in the rat than in the rabbit (0.21 vs. 0.08 synapses/fiber/day in the

diaphragm and 0.3 vs. 0.14 synapses/fiber/day in the soleus (Brown et al., 1976; Rosenthal and Taraskevich, 1977; see Table I). In the rat, the soleus muscle lags the diaphragm by no more than one day in the latter stages of synapse elimination (Brown et al., 1976; Rosenthal and Taraskevich, 1977), whereas in the rabbit there is a delay of a week or more in the time of onset and of several days for the time of completion of the process. These observations are consistent with the facts that the motile period in the rat occupies only the last quarter of gestation (days 16-21) and that regional differences in other aspects of neuromuscular development are rather small in this species (East, 1931; Strauss and Weddell, 1940).

It is interesting to consider the possibility that the rate of synapse elimination is related to the ratio of motor neurons to muscle fibers in a muscle. This quantity, the "innervation ratio," varies significantly among different muscles in the same species (Porter and Hart, 1923; Eccles and Sherrington, 1930; Clark, 1931; Westerman et al, 1972; Warwick and Williams, 1973). Suppose, for example, that each motor neuron withdraws a fixed number of neuromuscular synapses per day, irrespective of the number of synapses on each fiber in the muscle. In this case, the rate of synapse loss per muscle fiber (which is the most straightforward way of describing our electrophysiological results) would be progressively greater for muscles with increasingly high innervation ratios. Another possibility is that the innervation ratio determines the peak level of multiple innervation. If individual motor neurons tend, on average, to establish the same number of peripheral synapses early in development, then muscles with a relatively high innervation ratio (the value presumably being fixed before the onset of synapse elimination) would have a correspondingly high maximal level of polyneuronal

innervation. That is, a high ratio of neurons to muscle fibers in association with a fixed number of synapses per neuron would imply a large number of synapses per muscle fiber. In this case, one would not necessarily expect a relationship between the innervation ratio and the rate of elimination, since the peak level of polyneuronal innervation may not be well correlated with the rate of synapse loss (e.g., EDL vs. pronator teres; see also Bennett and Pettigrew, 1974; Brown et al., 1976). Information on innervation ratios in rabbit muscles that could distinguish among these and other alternatives is not yet available.

An additional factor which might play a role in the elimination of synapses is neural and/or muscular activity. However, neuromuscular activity, as assessed by the presence of spontaneous and reflex-evoked embryonic movements, begins well before the onset of synapse elimination as determined by our techniques; we admittedly would not have detected an earlier stage of synapse loss if it were balanced by ongoing synapse formation. Moreover, we did not find any correlation between the time-course of synapse elimination and the contractile properties of different muscles, the differentiation of which begins around the time of birth (Close, 1964; Brown, 1973) and is influenced by the pattern of muscular activity (Fischbach and Robbins, 1969; Brown, 1973; Lømo, Westgaard, and Dahl, 1974). This suggests that the overall pattern of nerve or muscle activity is not the sole determinant of the timing of synapse elimination. Nevertheless, it remains a possibility that differences in activity of synapses innervating individual muscles play a role in determining which particular synapses are to survive.

Our results do not provide direct evidence concerning the specific factors regulating the removal of neuromuscular synapses, but

they do rule out simple hypotheses which depend only on synchronous, body-wide signals to initiate and control the process. It may be that peripheral synapse elimination is initiated by motor neurons according to a schedule determined by their position within the spinal cord. It is likely, however, that the overall time course of the process depends on specific interactions between an individual muscle and its neuronal inputs, inasmuch as synapse elimination can be delayed by procedures such as tenotomy (Benoit and Changeux, 1975) and partial denervation (Brown et al., 1976; Thompson and Jansen, 1977). It remains to be determined whether these interactions involve nerve or muscle activity patterns, selection by Schwann cells during myelination, sensory feedback to motor neurons, or other, as yet unrecognized factors.

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Chapter II

The Effects of Motor Unit Size on Innervation Patterns in Neonatal Mammals

The formation of supernumerary neuromuscular synapses and their subsequent elimination are now well-established phenomena in the development of mammalian skeletal muscle (Redfern, 1970; Bennett and Pettigrew, 1974; Brown, Jansen and Van Essen, 1976; Riley, 1976; Bixby and Van Essen, 1979). Similar processes occur during the development of the central nervous system (Conredi and Ronnevi, 1977; Crepel et al., 1976), and it is of general interest to elucidate the factors controlling the total number of supernumerary connections and the rules governing their loss. Previous studies have suggested that motor neurons in neonatal mammals have an intrinsic tendency to withdraw synapses, and that this tendency is greater for motor units with large peripheral fields, giving neurons belonging to smaller motor units an advantage in postnatal synaptic competitions (Brown et al., 1976; Thompson and Jansen, 1977). This is consistent with other lines of evidence suggesting that the size of the peripheral field is important in competitive synaptic interactions (Purves, 1976; Brown and Ironton, 1978).

One approach to this problem is to compare the development of different muscles in the same species, since it is known that the average size of motor units (i.e., the number of synapses/motor neuron) varies widely among muscles in the adult (Feinstein et al., 1955; McComas, 1977). Thus, one can ask whether there are corresponding disparities in the average size of motor units earlier in development, when polyneuronal innervation is present, or whether instead each motor neuron initially

tends to establish the same number of synapses. Similarly, one can ask whether the rate of loss of synapses per motor neuron during the synapse elimination period is constant, or whether this rate is dependent on other factors such as the size of the peripheral field or the extent to which individual muscle fibers are polyneuronally innervated. In an earlier study on synapse elimination in the neonatal rabbit (Bixby and Van Essen, 1979), we found differences between muscles in the average number of synapses per muscle fiber at the peak stage of multiple innervation. Evaluation of these data in terms of the peak number of synapses per motor neuron requires knowledge of the innervation ratio (i.e., the ratio of the number of α-motor neurons to the number of muscle fibers) for each muscle. Similarly, there are differences between muscles in the number of synapses lost per muscle fiber per day during the stage of most rapid synapse elimination (Bixby and Van Essen, 1979), but it is necessary to know the innervation ratios of the muscles involved in order to determine how the elimination rate per motor neuron varies.

In order to obtain this information we have made counts of motor neurons and muscle fibers for three different rabbit muscles: the soleus, the extensor digitorum longus (EDL), and the pronator teres (pronator). These muscles were chosen because information was already available concerning their respective levels of multiple innervation and rates of synapse elimination (Bixby and Van Essen, 1979). The results suggest that the average number of synapses per motor neuron is relatively constant early in development even though the final value varies considerably among muscles. On the other hand, the rate of synapse loss per motor neuron varies markedly but in a manner not closely related to the total number of synapses to be eliminated.

For muscle fiber counts, muscles were dissected from young (postnatal day 16) New Zealand White rabbits (Oryctolagus cuniculus), and fixed in Karnovsky fixative (Karnovsky, 1965). After post-fixation in OsO_4, dehydration, and Epon embedding, semi-thin (1 μm) sections were cut on glass knives, and counts were made directly from photographic montages of toluidine blue-stained cross-sections cut through the mid-portion of the muscle. Motor neuron counts were obtained from rabbits 9-14 days old by the technique of retrograde transport of horseradish peroxidase (HRP). For each muscle, the motor nerve was transected near its point of entry into the muscle, and pulled by a thread into a capsule made from polyethylene tubing sealed at one end. The capsule was filled with 3-4 μl of 40% HRP in mammalian Ringer's, and the open end was sealed with silicone grease. In most experiments ∿ 10 μg phospholipase A_2 was added to the HRP solution to inhibit sealing of the cut axons (E. Frank, personal communication). After 3-4 days, the animals were perfused with Karnovsky fixative, and the relevant regions of the spinal cord were removed, fixed for a further 3-4 hours, washed overnight in cold, buffered 30% sucrose, and sectioned at 40 μm on a freezing microtome. Sections were reacted for HRP activity according to Graham and Karnovsky (1966), or with a modified technique using $CoCl_2$ to enhance sensitivity (Adams, 1977), and lightly counterstained with cresyl violet. Counts were made from serial sections of all neurons containing reaction product, which were then separated into α and γ-motoneurons on the basis of average somal diameter; the histogram made from pooled data was clearly bimodal. Corrections for double counting were made using the method of Clarke, Rogers and Cowan (1976).

The number of muscle fibers in each of the three muscles is listed in Table I. For the soleus, counts made from two different muscles agreed within 5%, but in general the variation in muscle fiber number is probably closer to 10-15% (Thompson and Jansen, 1977; Frank, Jansen, Lømo and Westgaard, 1975).

The success of the HRP transport was variable, due to factors such as leakage from the capsule and displacement of the nerve. However, in several cases for each muscle, HRP-positive neurons were found in the spinal cord (Figure 1A). The EDL and soleus motor neurons are located respectively in spinal segment L7 and spinal segment S1, with an area of overlap at the border between these segments amounting to about 5% of the neurons. The neurons innervating the pronator are mainly in spinal segment C7, with a slight extension into C8. As is well known for other mammals (Romanes, 1964; Rapoport, 1978) the motor neurons for these rabbit muscles are organized into elongated cell columns (Figures 1B, C, D). Within these columns there is no apparent segregation of α- and γ-motoneurons.

The number of HRP-positive α-motor neurons varied considerably in different experiments on the same muscle, so the values used in estimating the total number of neurons in a given nucleus were the two highest counts obtained. Table I gives these values for each of the three muscles. To check on the accuracy of the HRP method, we obtained an independent estimate of the number of motor neurons for the EDL, by counting the number of large myelinated axons in the EDL nerve after ablation of dorsal root ganglion L7. The number of α-fibers estimated in this experiment was 90, which is in good agreement with the highest count obtained

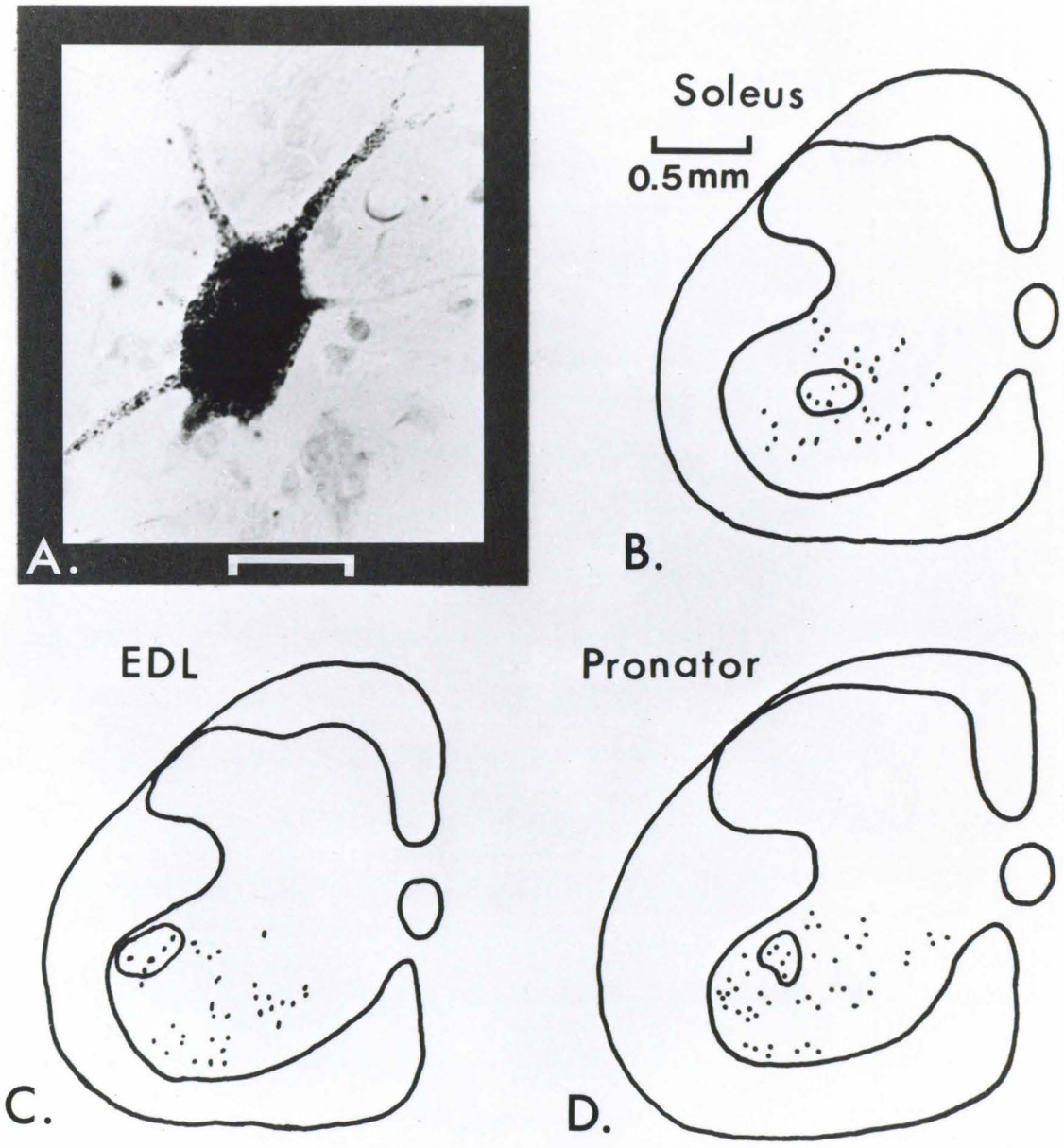
Table I

Muscle	No. of Muscle Fibers	No. of α-Motoneurons	Avg. Motor Unit Size ($\frac{\text{musc. fiber}}{\text{mot. neur.}}$)	Peak Innerv. Level (syn./m.f.)	Peak Rate of Synapse Elimination (syn./m.f./day)
Soleus	11,042; 11,508	65;65	170	3	0.14
EDL	33,052	87;90†	370	2	0.12
Pronator	4,598	18;18	255	2.2	0.06
Rat soleus	2,700*	24*	110	5*	0.30

*Data from Brown et al. (1976)

†Approximately 90% of the L7 ganglion was removed in an operation on an adult rabbit anesthetized with Nembutal. After a survival time of 10 days, the EDL nerve was dissected out, fixed, embedded in Epon, and sectioned at 1 μm for a fiber count. The number of Group I axons counted was 99, which is only an upper limit to the number of α-motor neurons due to the incompleteness of the ganglionectomy. We assumed that about 10% of these were surviving sensory axons, since the normal EDL nerve was found to have ca. 200 Group I axons, of which slightly more than half are therefore sensory.

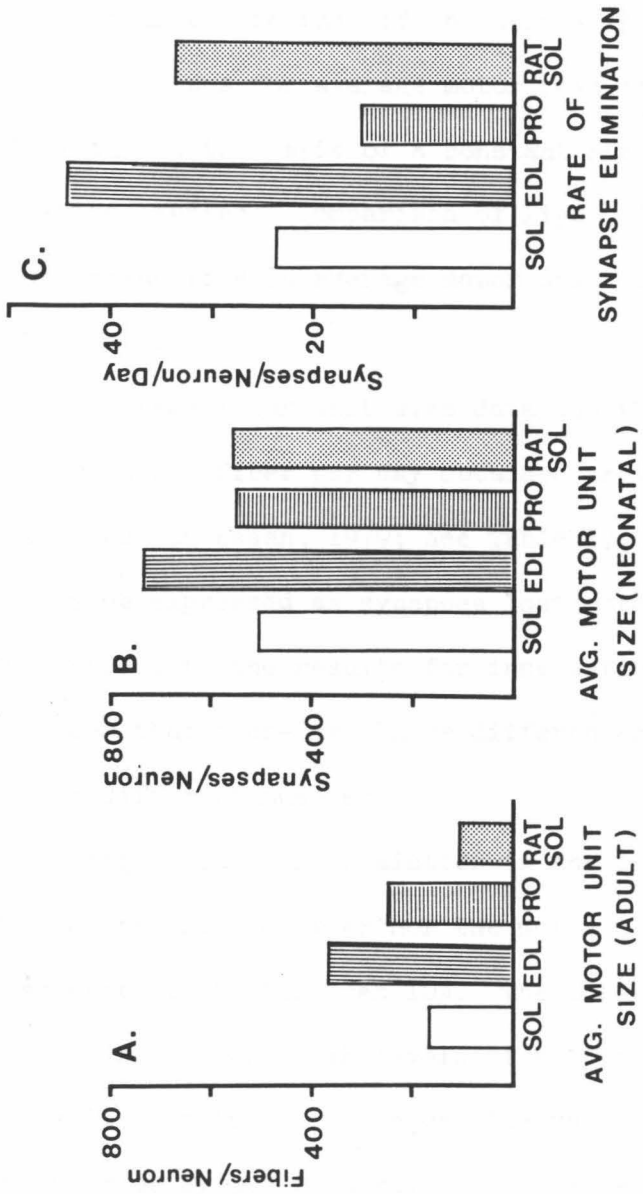
Fig. 1. A: Motor neuron in the ventral horn of the cervical spinal cord, labeled with HRP from the pronator nerve (DAB-$CoCl_2$ reaction). Bar = 25 μm. B, C, D: Cell columns in the spinal cord containing the motor neurons for the three muscles under study. Each drawing is a traced outline of half the spinal cord from a single representative section. The dots represent cells which stain darkly with Nissl stain in the sections whose outlines are traced, and the heavy lines enclosing groups of cells are the boundaries of the respective motor neuron pools, determined by aligning tracings of sections taken over the rostro-caudal extent of the labelled column. Bar in B gives the scale for all three tracings. B: soleus neurons. C: EDL neurons. D: Pronator neurons.


A.
Soleus
0.5mm
B.
EDL
C.
Pronator
D.

in the HRP experiments. Table I also lists the average motor unit size estimated for each adult muscle, which is repeated in Figure 2A.

Estimates of the peak levels of multiple innervation were made for the soleus and EDL by taking advantage of the existence of a plateau in innervation level for each muscle during the first 4 postnatal days (Bixby and Van Essen, 1979). The soleus estimate is based on measurements of motor unit tension overlap, while the EDL peak estimate of 2.0 is based on 90% of the fibers being multiply innervated during the plateau and about 10% of these being triply-innervated fibers (cf. Rosenthal and Taraskevich). For the pronator, the rate of synapse elimination is much lower, and we were unable to demonstrate a plateau. Thus the peak value we obtained at embryonic day 26 (E26) of 90% multiple innervation is a slight underestimate if synapse elimination has already begun by E26 - the earliest stage at which we were able to obtain data (Bixby and Van Essen, 1979). We therefore estimated the maximum level of multiple innervation as approximately 20% higher than that observed at E26. These values are listed in Table I. Also in Table I are the data for the rat soleus (Brown et al., 1976), which are included for a cross-species comparison. The average number of synapses made by each motor neuron during development can be computed from the data in Table I, and their magnitudes are illustrated in Figure 2B. It should be noted that large errors could arise if the number of motor neurons or the number of muscle fibers changed significantly after the time at which the measurements were made. The death of motor neurons is over by this time (Harris-Flanagan, 1969; Brown et al., 1976), but it has been

Fig. 2. A: Average adult motor unit size (No. muscle fibers/No. motor neurons) for four different mammalian muscles. There is more than a three-fold variation among the muscles examined. B: Average motor unit size for the same muscles during the peak period of multiple innervation. Note that the motor units are much larger at this time, and that the variation in the estimates for the four muscles is only 31%. C: Rate of synapse elimination expressed as synapses lost/motor neuron/day, for the four muscles. There is about a three-fold variation in the estimates for the different muscles. Abbreviations: SOL, rabbit soleus; EDL, rabbit extensor digitorum longus; PRO, rabbit pronator teres; RAT SOL, rat soleus.


A.
800
400
Fibers/ Neuron
SOL EDL PRO RAT SOL
AVG. MOTOR UNIT SIZE (ADULT)
B.
800
400
Synapses/Neuron
SOL EDL PRO RAT SOL
AVG. MOTOR UNIT SIZE (NEONATAL)
C.
40
20
Synapses/Neuron/Day
SOL EDL PRO RAT SOL
RATE OF SYNAPSE ELIMINATION

suggested that there is significant postnatal addition of muscle fibers in some muscles (Chiakulas and Pauly, 1965; Caldwell et al., 1979). We have evidence that this does not occur for the rabbit or rat soleus, however (Bixby and Van Essen, 1979; Thompson and Jansen, 1977), and it is likely that differentiation of the other rabbit muscles we have studied is comparable to that of the soleus (Bixby and Van Essen, 1979). Therefore, the values for average motor unit size in immature muscles were calculated on the basis of a constant number of muscle fibers over the period studied. Comparison of Figs. 2A and 2B suggests that there is less variability in average motor unit size in immature muscle than in the adult.

Using the average motor unit size data and the estimates of synapses lost per muscle fiber per day obtained previously (Brown et al., 1976; Bixby and Van Essen, 1979; see Table I), the synapse elimination rate can be expressed as synapses lost/motor neuron/day (Figure 2C). In contrast to the results for innervation levels, it can be readily seen that there are large differences in rate of loss of synapses for different muscles.

There are significant uncertainties in the data contained in Figure 2. Neither the muscle fiber nor the motor neuron count is likely to be accurate to better than 10%. The uncertainties in the rates of synapse elimination and peak levels of multiple innervation are also of comparable magnitude. Therefore the values in Figures 2B and 2C may be in error by 30% or more. Hence, these results, while suggestive, are not conclusive.

The data in Figure 2B suggest that motor neurons from muscles with a ten-fold range of fiber number tend initially to establish

an average of 500-700 neuromuscular synapses during development. Due to variation in the muscle fiber/motor neuron ratio, levels of multiple innervation vary by a factor of 2-3. A relatively constant average motor unit size is of interest in view of the wide range in size of individual motor units reported for the neonatal rat soleus muscle (Brown et al., 1976). This could reflect the existence of different mechanisms controlling synapse number within the muscle as a whole and that within individual motor units. Alternatively, it might be that the times at which peak synapse number is attained are different for different motor neurons. It is suggestive that this estimate of the number of synapses/motor neuron agrees well with the limit of 550 obtained for sprouting in partially denervated adult soleus muscle (Thompson and Jansen, 1977). This supports the idea that for muscles of moderate size there is an intrinsic limit of about this magnitude to the number of synapses that their motor neurons can support. It is clear that some mammalian motor neurons are capable of maintaining more synapses, in muscles with a large number of muscle fibers (McComas, 1977). Conversely, there is evidence that, for very small muscles, motor neurons need not form as many synapses during the peak innervation period (Betz, Caldwell, and Ribchester, 1979). Thus the results are indicative not of a strict constancy of average neonatal motor unit size, but rather less variability at this time than in the adult.

The reasons for the variations in rate of synapse elimination are unknown. One possibility is that the amount and/or pattern of neuromuscular activity is important in synapse elimination (Benoit and

Changeux, 1975, 1978; Thompson et al., 1979), and that variations in overall activity patterns among muscles result in varying elimination rates. Another is that the rate of elimination depends on the magnitude of whatever differences exist in competitive ability between synapses coexisting on individual fibers. In this case, synapses would be lost rapidly from fibers on which one synapse had a large competitive advantage, and slowly from fibers on which all synapses were on an equal basis. The basis of these competitive interactions might be activity differences between different neurons within a muscle, or might be related to the competitive advantage which apparently exists for synapses belonging to small motor units over those in large motor units. In the latter case, large variations in neonatal motor unit size would lead, on average, to synapses of unequal strength coexisting on muscle fibers, which could lead in turn to a high synapse elimination rate. Thus differences among muscles in the total range of motor unit sizes, if such differences exist, could lead to the observed variations in elimination rate.

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Chapter III

Ultrastructural Observations on Synapse Elimination in Neonatal Rabbit Skeletal Muscle

Much of our understanding of synaptic structure and function stems from research on the vertebrate neuromuscular junction (NMJ). There has been considerable interest in establishing the sequence of developmental events leading to the precisely organized structure of mature synapses. Early ultrastructural studies of mammalian neuromuscular development were mainly concerned with the sequence of events involved in the formation of synapses by the immature motor axons, and regarded the situation in early postnatal mammals as essentially representative of adult terminal morphology (Teräväinen, 1968; Kelly and Zacks,1969). Subsequent physiological studies demonstrated an unexpected degree of complexity in neuromuscular development, however, in that neonatal mammalian muscle fibers go through a transient stage of multiple innervation before reaching the mature, singly-innervated state (Redfern,1970; Bennett and Pettigrew, 1974; Riley, 1976; Bixby and Van Essen, 1979). This discovery focussed attention on a different aspect of synaptic development--the elimination of supernumerary synapses. This elimination occurs without a decrease in the number of motor axons, indicating that each motor neuron loses much of its initial complement of synapses. Whether this loss occurs by degeneration of synapses or their retraction into the parent axon has been examined in two electron microscopic studies. Korneliussen and Jansen (1976) found no degenerating terminals at endplates examined during the peak period of synapse elimination, and suggested accordingly

that most synapses were removed by retraction. Rosenthal and Taraskevich (1977), on the other hand, reported a high incidence of degenerating terminals in their material, and concluded that degeneration similar to that occurring in adults following nerve transection is the predominant mechanism of synapse elimination.

One of the goals of the present study was to reexamine this issue of degeneration vs. retraction during maturation of neuromuscular connections. Though a large percentage of the preexisting synapses disappears over a period of just a few days of development, it is not obvious what the likelihood is of observing signs of degeneration in a large population of synaptic profiles, should synapse elimination in fact involve degeneration. This probability depends on the amount of time over which individual degenerating synapses persist in neonatal muscle, as well as the dimensions of a degenerating terminal relative to those of an entire endplate. In order to obtain such information, the appearance of neonatal synapses was examined electron-microscopically at various times following nerve transection. This proved to be important because both the time course and sequence of ultrastructural changes following neonatal nerve section differ somewhat from those in the adult.

Several lines of evidence suggest that synapse elimination is not a random process, and that the determination of which synapses are to survive involves interactions between terminals coexisting on individual muscle fibers (Brown, Jansen, and Van Essen, 1976; Thompson and Jansen, 1977). It is not clear, however, whether these interactions are direct or whether they involve the other cells in the vicinity; i.e., Schwann cells or muscle fibers. One simple hypothesis, for

example, is that terminals are selected for survival by the myelination of their pre-terminal axons (Brown et al., 1976). It is well known from previous work that myelination in peripheral nerves occurs around the time of birth (Webster, 1971; Peters and Muir,1959; Friede and Samorajski,1968), and in the present study the extent to which myelination has progressed along the intramuscular nerve branches was examined. The evidence suggests that myelination per se is not involved in the elimination of neuromuscular synapses.

Materials and Methods

Experiments were performed on New Zealand White rabbits (Oryctolagus cuniculus), ranging in age from 0.5 to 3 days. For observation of normal material, diaphragms were quickly dissected from animals anesthetized with ether, pinned out in oxygenated Ringer's solution, and immediately fixed in a solution containing 2.5% glutaraldehyde, 1.5% paraformaldehyde, and 0.1 M phosphate buffer, pH 7.4. In one case, the muscle was stained for acetylcholinesterase (Karnovsky and Roots, 1964) after 10 minutes in fixative. Blocks were cut from the midportion of the diaphragm, fixed for a further 3-4 hours, postfixed in buffered 1% OsO_4, washed in buffer, and dehydrated through a graded series of ethanols. En bloc staining with uranyl acetate in 95% ethanol was usually performed. Following Epon embedding, blocks were trimmed and sections transverse to the muscle fibers were cut on glass (semi-thin sections) or diamond (thin sections) knives on an LKB ultramicrotome. Grids were stained with uranyl acetate and lead citrate.

For denervation studies, the left phrenic nerve was cut close to the point of entry to the diaphragm in rabbits 0.5-1 day old anesthetized

with ether. The incision in the skin and muscle was closed with wound clips, and animals were allowed to survive for periods of 8, 9.5, 11, 13.5, 18, 22, and 25 hours (1 animal each) before sacrifice. Blocks were taken from both the left (experimental) and right (control) hemidiaphragms, within 0.5 to 1.5 cm of the nerve entry, and processed as described above.

Endplates were sampled using several different procedures. Several endplates were examined in serial thin sections. These sections were picked up on celloidin-coated grids, and approximately every 2nd section was photographed. Some sections were lost for technical reasons, but no more than four successive sections were missed in these series. Other endplates were examined in semi-serial sections, with approximately every 5th section being photographed. Most endplates were sampled in single cross-section, however. Care was taken to ensure that each endplate was photographed only once. Groups of 7-8 thin sections were taken, and at least 25 µm was cut from the block between groups of sections. One section from each group was chosen for detailed examination, but portions of other sections which revealed areas obscured by grid bars or debris in the chosen section were also examined. The axons giving rise to identified terminals were traced in several cases by cutting alternating series of semi-thin and thin sections. Finally, 104 endplates from experimental (denervated) hemidiaphragms and 33 endplates from control hemidiaphragms were observed in single cross-sections, using the sampling procedure described above (see Table I).

Table I

Number of endplates examined at various times after nerve section

Time (Hours)	No. of Experimental Endplates	No. of Control Endplates
8	10	11
9.5	9	4
11	9	-
13.5	39	5
18	12	4
22*	11	-
25	14	9

*The endplates examined at this time were taken from a region immediately adjacent to a nerve branch damaged during the operation, and thus are likely to be in an least as advanced a stage of degeneration as those examined at 25 hours.

Results

Morphology of normal endplates in cross-section

The ultrastructure of synapses in the neonatal rabbit diaphragm as seen in individual sections is generally similar to that described for comparable stages of development in the rat (Teräväinen, 1969; Kelly and Zacks, 1969; Rosenthal and Taraskevich, 1977), and will not be exhaustively described. The major difference between neonatal and adult neuromuscular junctions is the higher incidence in the neonate of multiple terminal profiles in single sections (Fig. 1). The post-synaptic folds are generally less well developed in the neonate, although there was considerable variation in the depth of these folds in the endplates examined in this study (Figs. 1, 2). In addition, the synaptic vesicles are fairly evenly distributed in neonatal terminals, with little increase in density near the synaptic cleft (Fig. 1). All of the endplates examined were taken from rabbits 0.5-3 days old, a period during which synapse elimination is at its peak in the diaphragm (Bixby and Van Essen, 1979). Though there are differences between newborn and 2-day-old animals in the proportion of endplates in various stages of maturation, the general descriptions presented here are valid for the entire developmental window under consideration.

Degeneration of terminals following nerve section

Neonatal muscles were examined at various times after denervation in order to determine the time course and appearance of terminal degeneration at a time when synapse elimination is normally occurring. 7 muscles were examined between 8 and 25 hours following nerve section. All 10 of the endplates examined 8 hours after denervation had a normal appearance (Fig. 2). The first abnormalities in terminal structure

were seen at 9.5 hours following nerve section (6 of 9 endplates abnormal), while the last endplates to exhibit normal ultrastructure were seen at 13.5 hours after denervation (2 of 39 endplates normal). All endplates examined from the 18-, 22-, and 25-hour denervated muscles exhibited markedly altered structure. There is some variability among muscles with respect to this time course, as the endplates examined at 11 hours all had a virtually normal appearance (Fig. 3), and were thus less advanced than those seen at 9.5 hours. These data indicate that there is a lag period of 9-11 hours following nerve transection before changes are visible at motor nerve terminals, and that there is a period of less than 5 hours during which virtually all synapses become degenerative.

The most consistent feature of the early stages of degeneration was the separation of terminals from the muscle fiber membrane by fingers of Schwann cell cytoplasm (Figs. 4, 5). In many cases this was the only apparent structural abnormality. In addition, no cases were observed of terminals with abnormal structure directly apposed to the muscle fiber. Taken together, these observations indicate that the initial event in neonatal terminal degeneration following nerve section is the glial investment of terminals. This differs from the adult, in which degenerating terminals are frequently (but not invariably) seen directly opposite the muscle fiber (Miledi and Slater, 1970; Manolov, 1974; Winlow and Usherwood, 1975).

Actual degenerative changes within terminals had also begun by 9.5 hours and were evident at the majority of endplates by 13.5 hours after denervation. The signs of degeneration were similar to those described by other investigators (Miledi and Slater, 1970; Manolov,

Plate 1

Fig. 1. Normal morphology of synapses in the neonatal rabbit diaphragm (1.5 days postnatal). Several terminal profiles, not separated by the Schwann cell, overlie a single muscle fiber. A, axon; M, muscle fiber; NT, nerve terminal; SC, Schwann cell. X24,500. (Bar = 1 μm).

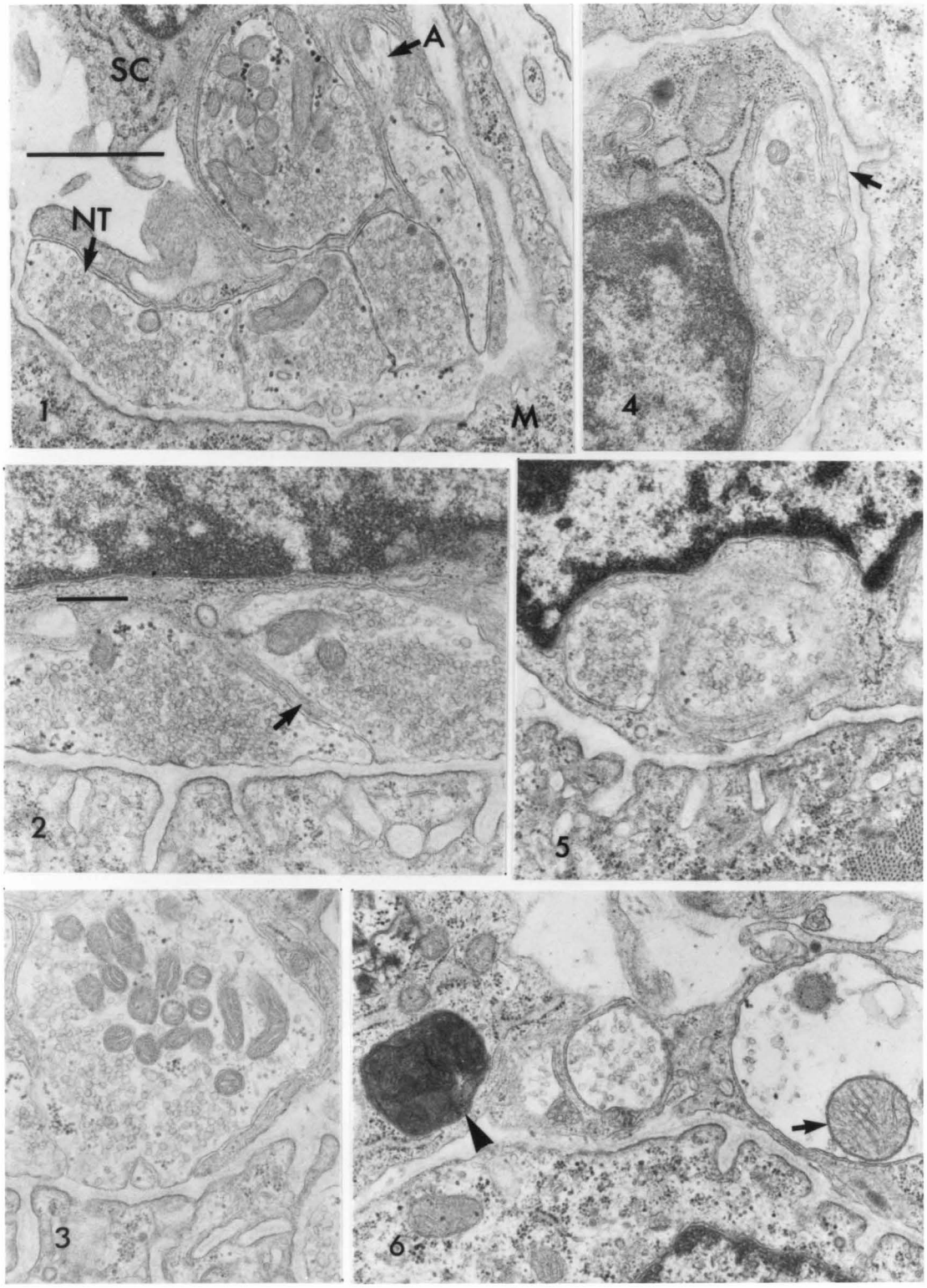
Fig. 2. NMJ from a neonatal muscle denervated 8 hours before fixation. Appearance is quite normal. Arrow indicates glial finger separating two terminal profiles. X25,400. (Bar = 0.5 μm for Figs. 2-6).

Fig. 3. NMJ from a muscle denervated 11 hours before fixation. Appearance is normal. X25,400.

Fig. 4. NMJ from a muscle denervated 9.5 hours before fixation. Appearance is normal, except for Schwann cell fingers separating terminal from muscle fiber (arrow). X25,400.

Fig. 5. 9.5 hours post-denervation. Two normal-looking terminal profiles are completely enwrapped by the Schwann cell. X25,400.

Fig. 6. 9.5 hours post-denervation. 3 neuronal profiles are engulfed by the Schwann cell. One profile is fairly normal, one exhibits clustering and sparsity of synaptic vesicles, as well as a swollen mitochondrion (arrow), and the third is completely dense (arrowhead). X25,400.


SC
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NT
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1974; Winlow and Usherwood, 1975) including swollen and damaged mitochondria (Fig. 6), clumping and loss of synaptic vesicles (Figs. 6, 7), increases in cytoplasmic density (Figs. 6, 7, 8), "honeycomb" structures (Fig. 7), and dense lysosome-like inclusions in Schwann cells. Combined with the absence of degeneration at 8 and 11 hours, the observation of many of these abnormalities at 9.5 hours indicates that severe degeneration can occur within a few hours.

An unexpected finding was that many junctional profiles examined at 13.5 hours or later after denervation contained neither synaptic terminals nor degenerating debris (Fig. 9). This was the case in 40% of the sections through endplates from the 13.5 hour muscle, and the majority of such sections from muscles taken 18 hours or more after denervation. These findings suggest that there is either a rapid sequestering of debris from degenerating terminals in neonatal muscle, or a retraction of part of the terminal from the endplate before Schwann cell engulfment.

In order to assess the likelihood of seeing degeneration due to normal synapse elimination, it is necessary to know the time integral of the probability of seeing either frankly degenerating terminals or degenerating debris in Schwann cells, as sections containing engulfed but otherwise normal profiles are not specifically indicative of degeneration. This exact information cannot be derived, but an estimate can be made from the available data. Profiles exhibiting degeneration were seen at 4 of 9 endplates examined at 9.5 hours, 19 of 39 at 13.5 hours, 5 of 12 at 18 hours, 1 of 11 at 22 hours, and 5 of 14 at 25 hours. A reasonable approximation, then,

Plate 2

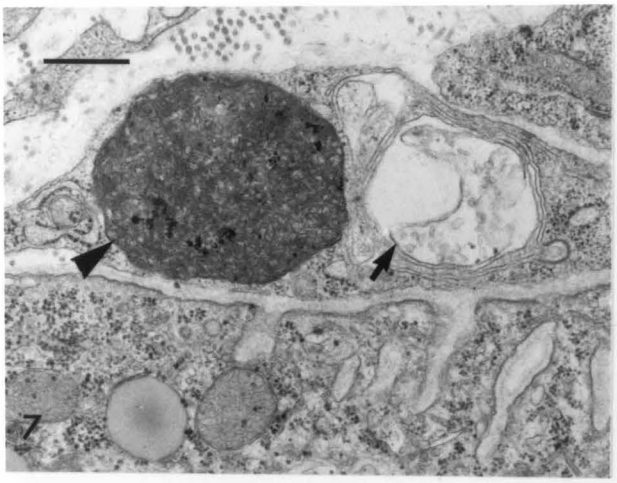
Fig. 7. 13.5 hours post-denervation. A Schwann cell engulfs one profile with markedly altered morphology (arrow), and one profile resembling the "honeycomb" structures described by other investigators (arrowhead). X25,400. (Bar = 0.5 μm for Figs. 7-11).

Fig. 8. 13.5 hours post-denervation. The Schwann cell contains dense debris and particulate material resembling glycogen. X25,400.

Fig. 9. 13.5 hours post-denervation. A Schwann cell containing no degenerating terminals or debris overlies junctional membrane. X25,400.

Fig. 10. 18 hours post-denervation. A Schwann cell free of debris and covering junctional membrane can be seen to wrap a normal-looking axonal profile (arrow). X25,400.

Fig. 11. 9.5 hours post-denervation. A normal-looking axon that was found a few μm away from an endplate with a badly degenerated terminal. X25,400.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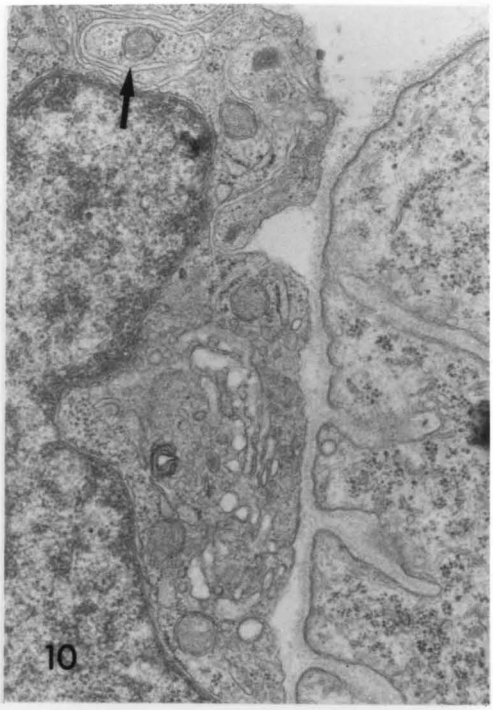
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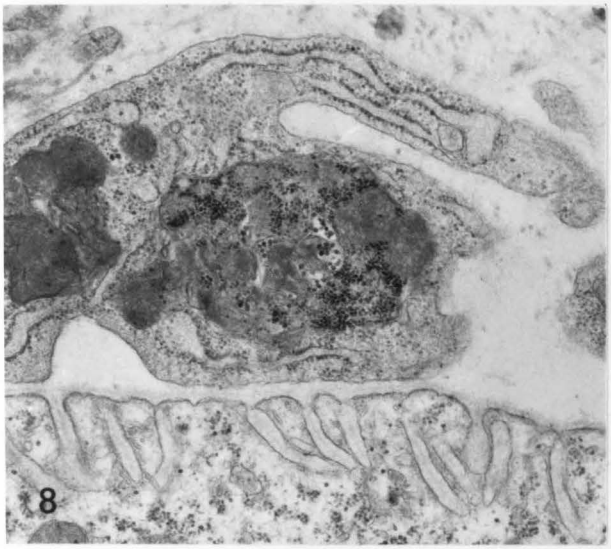
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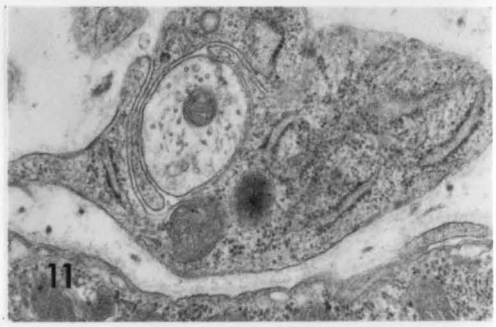
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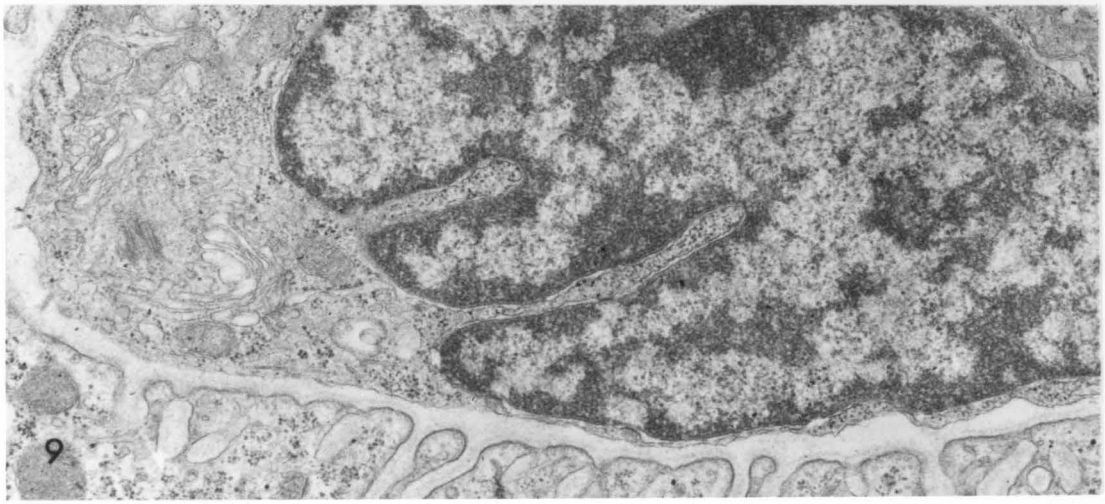
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is that signs of degeneration should be visible in about 40% of the sections through endplates at which a synapse is being lost over a period of 15 hours.

The pre-terminal axons exhibited a somewhat slower onset of degeneration than the terminals. Occasionally, at the early stages of terminal degeneration (9.5-13.5 hours), normal-looking axonal profiles were seen very close to endplates whose terminals were in advanced stages of degeneration, or even absent from the section (Figs. 10, 11). Farther back along the axons, in intramuscular nerve branches, clear degenerative changes were seen for the first time at 13.5 hours following nerve section. At this time, about half the axons observed appeared to be in normal condition (Fig. 12). This contrasts with the state of the terminals at 13.5 hours, since only 3 of the 39 endplates examined had terminal profiles whose ultrastructure was normal. By 25 hours after denervation, all of the axons in intramuscular branches were severely degenerated.

Absence of degeneration at normal endplates

In single cross-sections of 256 endplates examined from normal muscles of animals 0.5 to 2.5 days old, no degenerating terminals were seen. This was also the case for the 10 terminals examined over most of their length in serial or semi-serial cross-sections. This absence of degeneration is consistent with the findings of Korneliussen and Jansen (1976) in neonatal rat soleus, but contrasts with the findings of Rosenthal and Taraskevich (1977) on the rat diaphragm. In view of the results obtained with nerve section, it is important to note that the Schwann cells at these endplates were also examined for degenerating engulfed profiles or debris. No signs of degeneration

were found, the only abnormalities being small myelin figures in 10 of the 256 Schwann cells. These are a common artifact of aldehyde fixation, and were somewhat more common extracellularly than in glia. Moreover, the degeneration seen after nerve section is entirely different in appearance. Clearly the results provide no evidence for the presence of degeneration during normal development, but the degree to which this possibility can be ruled out will be considered in the discussion.

Observations on neuromuscular junctions in serial sections

In initial observations on single cross-sections, it was common to see vesicle-filled neuronal profiles that were enwrapped by glia and separated from the muscle fiber membrane by 1 μm or more. In order to explore the identity of these profiles, 3 endplates were examined in serial cross-sections along most or all of their length, and 7 endplates were sectioned semi-serially. These included both singly- and doubly-innervated endplates, a distinction which could usually be made by tracing axonal profiles as they left the endplate. In 8 endplates, irregular protrusions of the terminals were seen which extended above the surface of the muscle fiber for 0.5 to 5 μm before ending blindly. This length is substantial, considering that each terminal was only 10-20 μm long. Figs. 14-17 illustrate the appearance of a small terminal extension seen in successive cross-sections, and Fig. 13 is a serial reconstruction of a portion of an endplate exhibiting several large protrusions. There was considerable variation in the size and shape of these protrusions, as well as the distance they rose above the muscle; the terminal which is reconstructed in Fig. 13 is one of the more extreme examples. Instances of such terminal extensions were seen at both singly- and doubly-innervated endplates.

Plate 3

Fig. 12. 9.5 hours post-denervation. Several normal-looking axonal profiles (arrows) and one degenerating profile (arrowhead) in an intramuscular nerve branch. X25,400. (Bar = 0.5 μm for Figs. 12 and 18).

Figs. 14-17. Sections from a series through an NMJ that exhibited several irregular protrusions of the nerve terminal. The terminal profile in Fig. 14 becomes underlain by glial fingers (Figs. 14 and 15, arrows), and eventually moves somewhat away from the muscle fiber and is completely enwrapped (Figs. 16, 17). Arrow in Fig. 17 points to the last vestige of this terminal extension. The total distance covered by Figs. 14-17 is ∿ 1.5 μm. X17,800 (Bar in Fig. 14 = 1 μm).

Fig. 18. Several terminal profiles separated by glial extensions and a muscle fiber protrusion (arrow) at an NMJ from a 1.5-day-old rabbit diaphragm. A, pre-terminal axon. X25,400.

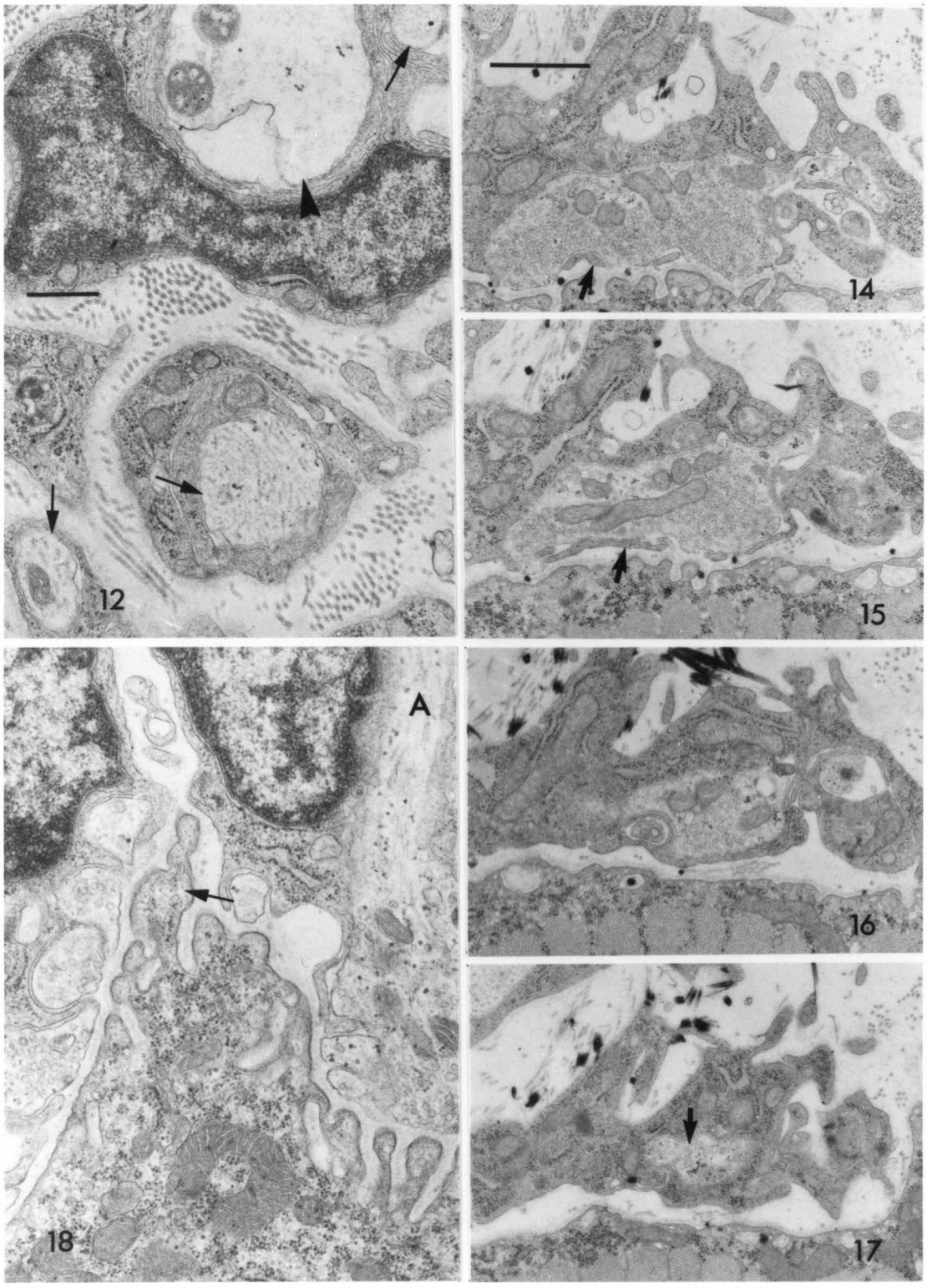
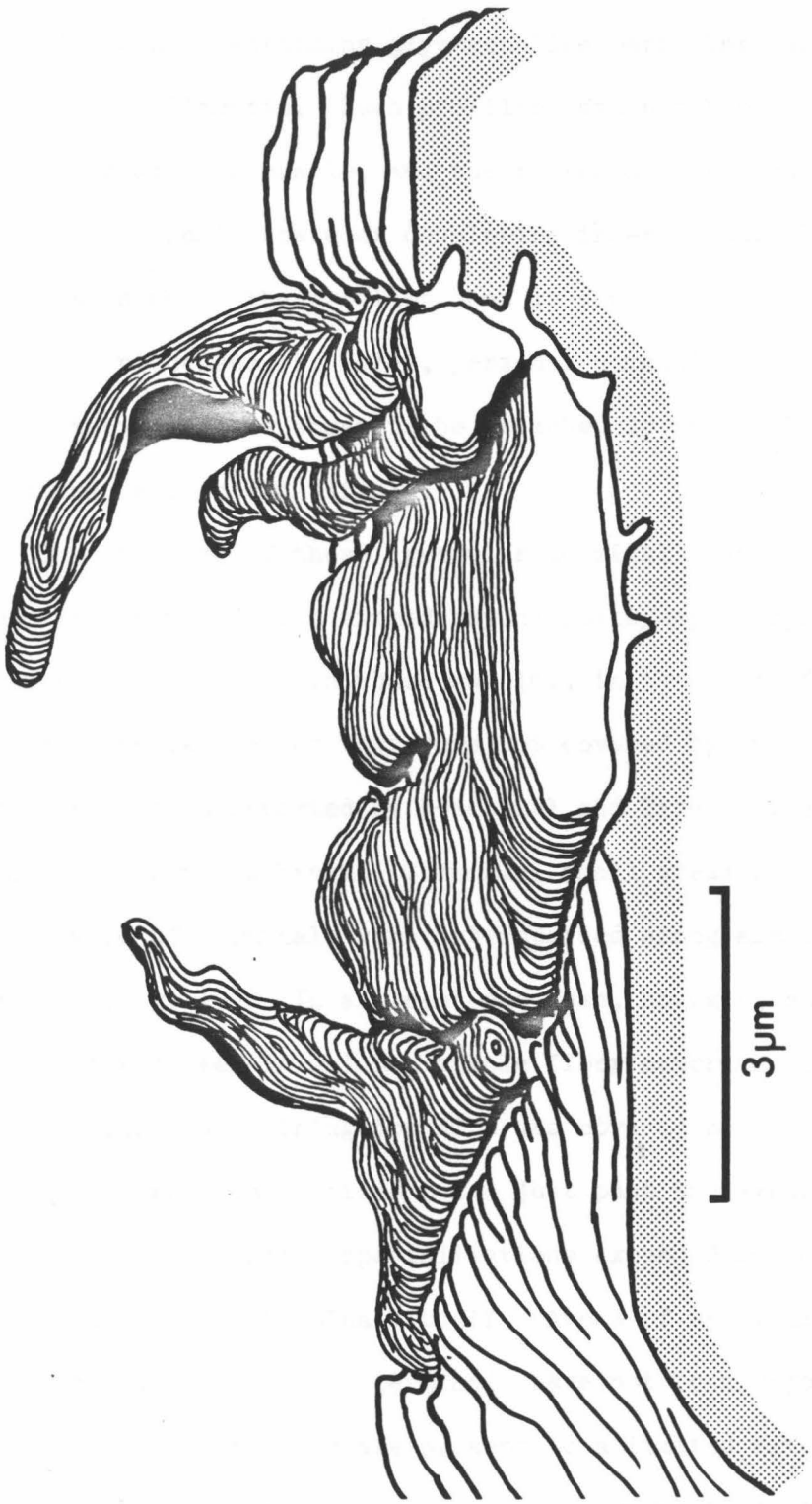

12
14
15
A
16
17
18

Fig. 13. A reconstruction of a portion of a neonatal nerve terminal, made from serial thin sections. The reconstruction was made by tracing the outlines of neuronal profiles from 10 evenly-spaced sections, spacing successive contours such that the final drawing is to scale. Three protrusions of the terminal can be seen to rise above the muscle fiber, in one case for 3-4 μm.


3µm

Their contents were indistinguishable from those of the synaptic terminals proper, including close-packed 35-50 nm clear vesicles, abundant mitochondria, densely-staining glycogen-like particles, and occasional microtubules or filaments. Such profiles have not been reported in normal adult mammalian muscle, and their presence here may well be related to the dynamic state of developing innervation. It should be noted that some of the profiles seen in single cross-sections may be retraction processes (D. Riley, personal communication), but in many cases they could be shown to be attached to terminals rather than to axons, and to end blindly.

The presence of these irregular terminal protrusions indicates that neonatal terminals can extend beyond postsynaptic specializations. The reverse was also seen in these muscles, in the form of areas of postsynaptic specialization that were not covered by synaptic terminals. These have also been reported by Rosenthal and Taraskevich (1977) in the neonatal rat diaphragm. Commonly, small areas of junctional membrane devoid of terminals were interspersed among areas of axonal terminations (Fig. 19). In at least one case, however, an entire cross-section was seen in which a muscle fiber membrane region containing junctional folds and staining for AChE was covered only by a Schwann cell. This occurred in sections taken just past the synapse on that fiber, and the postsynaptic specializations extended no more than 0.5 μm beyond the axon terminal itself. Areas of specialized junctional membrane not covered by nerve terminals have not been reported in adult mammals, although they are present to a limited extent in adult frog muscle (Letinsky et al., 1976). Their presence in neonatal mammalian muscle may be linked to the synapse elimination process.

Other investigators have noted that multiple terminal processes, which initially are in direct apposition, become segregated by fingers of Schwann cell cytoplasm (Kelly and Zacks, 1969; Korneliussen and Jansen, 1976; Atsumi, 1977). This suggests a possible role of Schwann cells in regulating terminal morphology; for example, by preferentially segregating terminals from different parent axons. In the neonatal rabbit diaphragm, axonal profiles were commonly separated by glial fingers (Fig. 18). Examination of serial sections through endplates revealed that terminals belonging to separate axons were often ensheathed by the same Schwann cell. In addition, though terminals from different parent axons at the same endplate were sometimes segregated by glia, many examples were seen of terminal profiles which were initially separated by Schwann cells, but which coalesced into a single terminal on nearby sections. As has been reported previously in other muscles (Kelly and Zacks, 1969; Korneliussen and Jansen, 1976; Atsumi, 1977), terminals could also be separated by protrusions of the muscle fiber (Fig. 18), but this did not appear to depend on whether the terminals segregated were from the same or different parent axons. There is thus no evidence that preferential separation of axon terminals occurs at neonatal endplates.

Relationship of motor axons to Schwann cells

One of the major goals of this study was to obtain evidence on the extent of myelination of intramuscular axons in order to assess the possibility of a role for glial cells in synapse elimination. In order to trace axons from their terminations back to the parent axons in the intramuscular nerve branches, alternating groups of semi-thin (1 μm) and thin serial sections were cut through several blocks.

This method allowed comparisons to be made between axons terminating on singly- and doubly-innervated fibers, while also providing an assessment of the overall state of myelination of the motor axons.

The large axons in extramuscular branches of the phrenic nerve are almost all heavily myelinated by the first postnatal day (Fig. 20). In the intramuscular nerve branches, however, axons were found in various stages of maturation, including partially-wrapped axons (Fig. 21), axons wrapped by a single turn of glial cytoplasm (Fig. 22), non-compact myelin (Fig. 23), and varying thicknesses of compact myelin (Fig. 23). The axons wrapped by compact or non-compact myelin were, in general, larger than those wrapped by only a single turn of glial cytoplasm. This is similar to the situation found in an earlier stage of development in rat peripheral nerve (Friede and Samorajski, 1968; Webster, 1971). Thus, the myelination process is ongoing in intramuscular nerve branches during the peak period of synapse elimination.

Although axons in the process of myelination were prevalent in intramuscular branches, it is necessary to know where the earliest stages of myelination are in relation to the synaptic terminals in order to assess whether this process is correlated with synapse elimination. If, for example, axons at singly-innervated endplates were myelinated, while those at multiply-innervated endplates were not, or if only one of the axons at a doubly-innervated endplate was myelinated, this would provide circumstantial evidence that Schwann cells might select axons for survival. With this in mind a small group of axons in one block was followed which had made synapses on fibers identified in semi-serial sections. Those axons remained unmyelinated

Plate 4

Fig. 19. A portion of specialized junctional membrane (between arrows) not covered by a nerve terminal. Muscle stained for AChE; arrowhead points to some reaction product. (2.5-day-old rabbit) X25,400. (Bar = 0.5 μm, Figs. 19-26).

Fig. 20. A myelinated axon from the phrenic nerve of a 1-day-old rabbit. Arrow points to myelin visible on a neighboring axon. X25,400.

Fig. 21. Axon in small intramuscular bundle from 2.5-day-old rabbit diaphragm. The Schwann cell does not wrap the axon completely. X25,400.

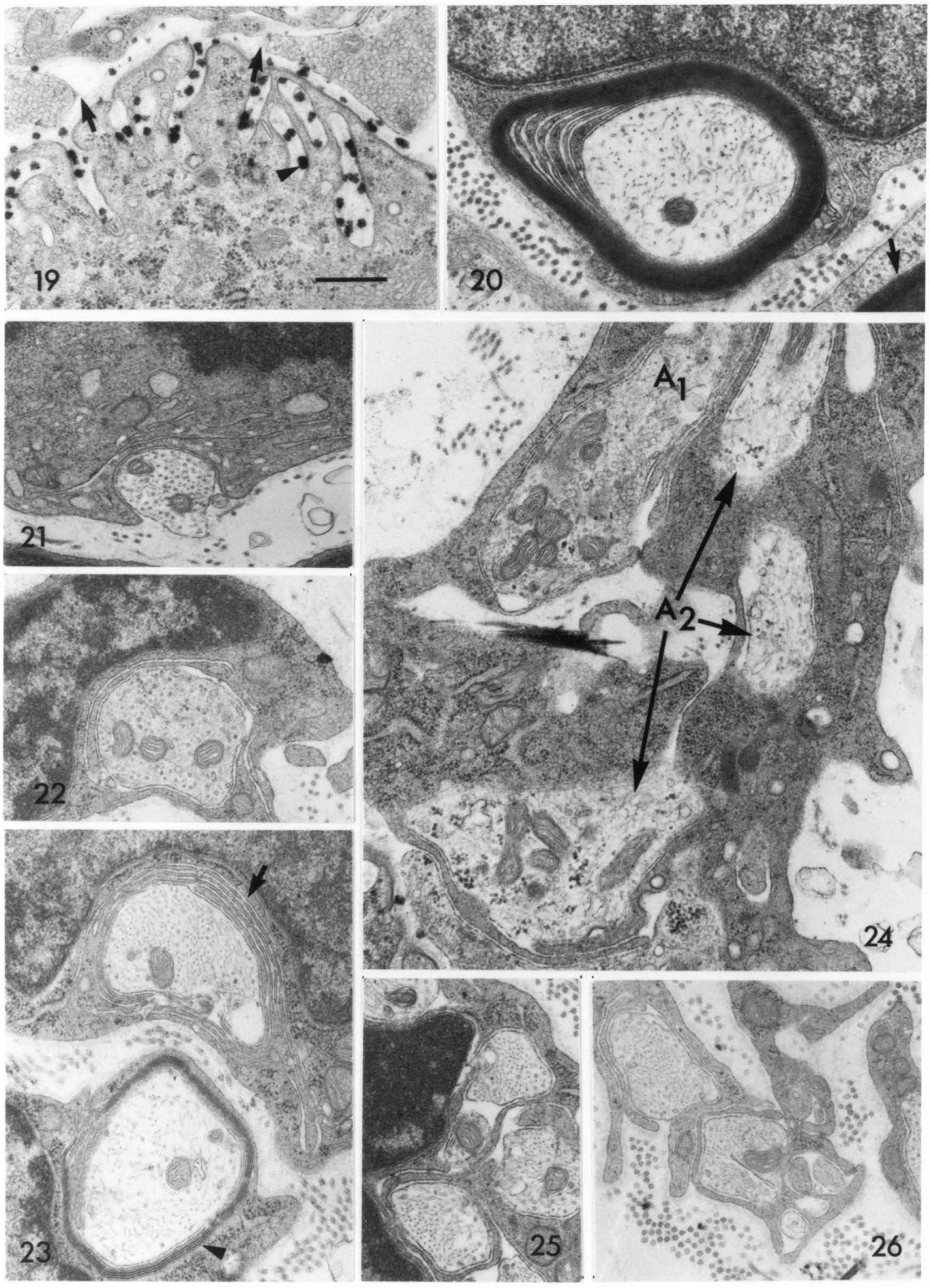
Fig. 22. Axon from 2.5-day-old rabbit diaphragm, showing complete wrapping by Schwann cell. X25,400.

Fig. 23. 2 axons in intramuscular nerve branch from 2.5-day-old rabbit. 1 is wrapped by several layers of non-compact membrane (arrow), and 1 shows several turns of compact myelin (arrowhead). X25,400.

Fig. 24. 2 axons (A_1, A_2) innervate a single muscle fiber. Some AChE reaction product is visible in lower left. A single Schwann cell invests both axons. X25,400.

Fig. 25. Several axons wrapped by a single Schwann cell, $\sim$ 15 μm from their terminations. X25,400.

Fig. 26. Several axons wrapped by a single Schwann cell, $\sim$ 150 μm from their terminations. X25,400.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for the entire distance they were followed (> 90 μm), after which they joined a large intramuscular nerve branch and could no longer be identified unambiguously. Among the endplates innervated solely by this group of axons were 2 identified as singly-innervated and 4 identified as doubly-innervated. In one case a single axon was identified as branching to terminate on both a singly- and a doubly-innervated muscle fiber. Thus, neither of the two possibilities suggested above is the case. Instead, it is apparent that pre-terminal axons can undergo synapse elimination at some branches and not others, while remaining unmyelinated.

In another block a group of 28 axons was followed distally in semi-serial sections for a distance of more than 220 μm. In this case, individual synapses were not identified as single or double, but the total number of endplates observed to be innervated by these axons over the length examined was 85, which means that the axons branched at least threefold on average. These axons were unmyelinated for this entire distance. Therefore, if selection by myelination is part of synapse elimination, this selection must take place at points on the motor axons more proximal than their last several branch points. This would imply that all the distal branches of some axons in a given sector of a muscle would survive, while other axons would lose all their branches in this sector. Such a situation would be expected to lead to the denervation of some muscle fibers, at least transiently, by a purely random process. Existing physiological (Brown et al., 1976; Bixby and Van Essen, 1979) and anatomical evidence suggests, however, that this occurs rarely if at all.

The relationship of individual pre-terminal axons to Schwann cells was examined in serial and semi-serial sections, to see whether evidence could be obtained for a "sorting-out" process at this level. Although in the mature state, each glial cell wraps only one axon, single Schwann cells commonly wrapped several axons in neonatal muscles at this stage. Examples were seen of two axons innervating the same end-plate which were wrapped by the same Schwann cell (Fig. 24). In addition, axons making synapses on both singly- and doubly-innervated fibers often were seen to travel together in small nerve bundles invested by a single glial cell. In the large group of axons that was followed for more than 200 μm, there was no discernible change in the morphological axon-glia relationship over this distance, the nerve fibers being wrapped by a single turn of cytoplasm (Figs. 25, 26). This distance probably corresponds to the territory of 3-5 Schwann cells for such immature nerve branches (Webster, 1971). These observations provide no evidence that the development of myelination in motor axons is correlated with synapse elimination, since final elimination decisions can be made by axons which are still immature in their relationship to the Schwann cells.

Discussion

The events following phrenic nerve section in neonatal rabbits are similar in many respects to those reported for adults of other species (Miledi and Slater, 1970; Nickel and Waser, 1968; Manolov, 1974; Winlow and Usherwood, 1975; but there are significant differences. The delay between nerve section and early degenerative changes is about 8-10 hours in the adult rat (Miledi and Slater, 1970), which is comparable to that described here for neonatal rabbits. Other investigators

(Manolov, 1974; Winlow and Usherwood, 1975) have suggested that swollen and damaged mitochondria are prevalent in terminals at earlier times after adult denervation; since abnormal mitochondria were also seen in control muscles in these studies, however, such changes may reflect an increased susceptibility of operated muscles to osmotic shock during fixation. The morphological signs of terminal degeneration are similar in adults and newborns, and most terminals have completely degenerated by one day or so after denervation in both cases. However, the time course of terminal degeneration in neonates differs in two respects from that reported for adults. First, Schwann cell profiles replacing nerve terminals and completely lacking in degenerating debris have not been reported in adults until ∿20 hours after denervation, while such profiles were common 13.5 hours after nerve section in the neonatal rabbits, and in the majority at all later times examined. Second, although there is considerable engulfment of terminals by glia at late times following adult denervation, substantial degenerative changes at least sometimes occur in terminals which are still apposed to muscle fibers (Miledi and Slater, 1970; Manolov, 1974; Winlow and Usherwood, 1975). This was not seen in denervated neonatal muscle.

There are at least two hypotheses which would explain the results obtained with neonatal denervation. First, it could be that the glial cells in newborn animals are faster and more efficient in phagocytosing denervated terminals than in the adult. This could explain the observation that Schwann cell profiles free of debris can be observed at relatively early times in the neonates. If Schwann cells in neonatal animals are more efficient at phagocytosis, however, the continued presence of debris in some glial cells at least 25

hours after denervation is puzzling. Perhaps an initial phase of engulfment and compaction by Schwann cells is more rapid in neonatal muscle, but the digestion of the remaining debris runs a time course similar to that in adult muscle.

An alternative to this scheme is that part of the initial response of neonatal terminals to separation from their somata is a retraction from the endplates. In this case degeneration could proceed along a time course similar to that following denervation in adult muscle, and no additional explanation would be required for the observed differences in the morphological changes associated with degeneration. The possibility that retraction of synaptic terminals occurs in this abnormal situation is of interest with respect to the hypothesis that such retraction occurs naturally during synapse elimination. This phenomenon could indicate that neonatal synapses are in a dynamic state in which each terminal is capable of retracting from its endplate under appropriate circumstances. The link between naturally occurring synapse elimination and the responses of nerve terminals to axotomy is obviously tenuous, however, and caution is required in interpreting these observations.

No degenerating terminals or degenerating debris in Schwann cells were seen at the endplates from normal muscle examined in this study. Interpretation of this result requires an estimate of the probability of observing degeneration in individual cross-sections, were it the primary mechanism involved. Such an estimate can be derived from the available data, with certain assumptions. The rate of synapse elimination in the neonatal rat diaphragm is 0.08 synapses/muscle fiber/day during the period examined here (Bixby and Van Essen, 1979).

This is actually a lower limit, since only the net loss of synapses was measured, and new synapses might still be forming during this period. The present study showed that degenerating debris can be observed in about 40% of the sections through denervated endplates over a period of about 15 hours. Thus the probability of seeing degeneration due to a synapse being eliminated would be 0.08 x 0.625 day x 0.4 of the sections, or 0.02 per section examined, assuming that the eliminated synapse originally occupied the entire length of the endplate being examined. This would be approximately true if the average size of synapses destined for elimination is the same as those that survive, owing to the considerable interdigitation between processes from different axons at an endplate. To allow for the possibility that the eliminated synapses are smaller on average, a correction factor of 0.7 can be introduced.[1] This would decrease the probability of seeing a terminal undergoing degeneration to 0.7 x 0.02 = 0.014 per section. Then the probability that one would not see degeneration in any one section would be 0.986, and the likelihood of examining 256 sections through different endplates without signs of degeneration would be $0.986^{256} < 0.03$. In addition, it should be noted that no degeneration was seen in the 10 endplates sectioned over most of their length. The evidence from this study, taken in conjunction with the lack of degeneration seen in rat soleus endplates by Korneliussen and Jansen (1976), and by Riley (personal

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[1] In the one double-innervated endplate examined in complete serial sections, one of the two synapses occupied only 26% of the synaptic area, but was visible over 72% of the total length of the endplate.

communication), strongly suggests that physical retraction is the primary means by which synapses are removed. The possibility cannot be ruled out, however, that some degeneration occurs during development, either as fragments lost from partially retracted terminals or as occasional synapses which degenerate completely.

In the one report claiming that degeneration occurs during normal synapse elimination, 13 of the 47 terminals examined during the peak period of elimination were reported to be abnormal (Rosenthal and Taraskevich, 1977). It is possible to compare this to the incidence expected on the basis of calculations like those just made. Since the rate of synapse elimination is approximately 2.5 times higher in the rat diaphragm than the rabbit (Rosenthal and Taraskevich, 1977; Bixby and Van Essen, 1979), the expected probability of seeing degeneration in any given section would be correspondingly higher, say 0.035 rather than 0.014. Even if the synapses to be eliminated were present along the entire length of the endplate, rather than 70%, this probability would increase only to 0.05. The expected incidence of degenerating material in 47 sections would therefore be 2, rather than 13. This is a significant difference ($p < .001$, χ^2 test). In addition the type of degeneration described by Rosenthal and Taraskevich (very condensed terminals still contacting the muscle fiber) is unlike that found in the present study to result when neonatal terminals are separated from their axons. Finally, these authors saw degeneration in 3 of 23 sections examined from a 21-day animal, a time when synapse elimination is virtually complete in the rat diaphragm (Bennett and Pettigrew, 1974; Rosenthal and Taraskevich, 1977). Evidently, then, some of the synapses destined to survive

had a highly abnormal appearance. It seems likely that those abnormalities were caused by the *in vitro* treatments to which the muscles were subjected.

The elongated, vesicle-filled extensions of immature nerve terminals seen in this study have not been reported previously. There is light microscopic evidence, however, which suggests that terminal extensions of this kind may be common at regenerating synapses, both in frogs (Letinsky *et al.*, 1976), and possibly in rats (Gutmann and Young, 1944; Jansen and Van Essen, 1975). Since such protrusions are either absent or rare in normal adult muscle, they are probably due to the rapid remodeling of synapses which occurs in neonatal animals. It could be that these terminal extensions represent portions of synapses being retracted, but one might expect them in this case to be near "naked" junctional membrane, which was often not observed. Conversely, they could be special structures resulting from the rapid growth of immature synapses. These structures are not simply manifestations of synapses being lost, since they occur at singly- as well as doubly-innervated endplates. As was pointed out in the Results section, their appearance in cross-section is similar to that of the synaptic terminals, but they also are similar in structure to axonal growth cones (Kawana, Sandri, and Akert, 1971; Yamoda, Spooner and Wessells, 1971; Landis, 1978). Though terminal extensions were common in these muscles, some of the profiles seen in single cross-sections which exhibited an extension-like morphology could belong to axonal processes which no longer end at synapses; i.e., "retraction bulbs" (D. Riley, personal communication), rather than these terminal extensions.

The observation that junctional membrane in neonatal animals is sometimes not covered by synaptic terminals supports a similar observation made by Rosenthal and Taraskevich (1977), using HRP-labeled α-bungarotoxin to label ACh receptors. Though clustering of ACh receptors can occur in the absence of nerve, junctional folds and AChE are thought to be induced in a muscle fiber by the overlying axon; the present results thus provide somewhat stronger evidence that the "naked" junctional membrane was once covered by axonal terminations. It is reasonable to suppose that these patches of membrane were once covered by synapses which have subsequently been lost, and that they are destined to become overgrown by the remaining axon terminal.

No direct evidence exists relating to the possible involvement of Schwann cells in synapse elimination. Brown et al. (1976) suggested as one of several possible mechanisms that synapse removal could involve the selection of terminals for survival by the myelinating glial cells. However, the observation that myelination has not reached the distal branches of motor axons during the peak elimination period argues against this possibility. The evidence is consistent with the view that myelination of the distal branches lags behind synapse elimination, and appears to be dependent, as in peripheral nerve (Matthews, 1968; Friede, 1972) on the diameter of the axonal process to be myelinated. Whether glial cells play any role in synapse removal is an open question. On morphological grounds, no evidence was found that Schwann cells distinguish among axons with respect to their stage of development in the elimination process. However,

the nature of the signal or signals which cause the loss of a synaptic terminal is unknown, and the axon could interact with both muscle and Schwann cell in making this decision.

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Chapter IV

Competition Between Foreign and Original Nerves in Adult Mammalian Skeletal Muscle[1]

It is well established that a normally-innervated, uninjured mammalian skeletal muscle does not accept further innervation from a foreign nerve transplanted onto the muscle's extrasynaptic region (Elsberg, 1917; Aitkin, 1950; Frank, Jansen, Lømo, and Westgaard, 1975). Since synapse formation under these circumstances would require the induction of new post-synaptic specializations, it is not clear whether foreign axons could make connections if they were allowed instead to compete for existing synaptic sites. The capacity of individual endplates to accept multiple inputs has in fact been demonstrated both during normal development (Redfern, 1970; Brown, Jansen, and Van Essen, 1976) and during reinnervation of adult mammalian muscle (McArdle, 1975; Benoit and Changeux, 1978); i.e., under circumstances involving interactions among recently formed synapses. We report here that even in adult muscles whose normal innervation remains fully intact, a similar susceptibility to further innervation can be expressed following appropriate placement of a transplanted foreign nerve. Moreover, the establishment of foreign synapses can lead to suppression of original inputs.

Under aseptic conditions a small motor branch of the superficial peroneal nerve was severed in 75-150 g Sprague-Dawley rats anesthetized

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with Nembutal. The proximal nerve stump was then transplanted directly over the endplate region midway along the soleus muscle. Care was taken not to injure the original (soleus) nerve or the muscle surface. After survival times of 1-14 weeks the muscle was removed along with foreign and original nerves for in vitro intracellular recording, as described elsewhere (Bixby and Van Essen, 1979). At the end of the physiological experiment, muscles were usually stained for acetylcholinesterase (Karnovsky and Roots, 1964) and/or nerve fibers and terminals (J. H. Steinbach, personal communication).

In the majority of experiments (61 of 104) the foreign nerve had drifted away from the placement site over the original endplate band; as expected, foreign innervation of soleus muscle fibers did not occur in these cases, with only rare exceptions. In 21 other experiments, the foreign nerve remained in the general vicinity of the original nerve but was only loosely attached to the muscle; foreign axons failed to grow into direct contact with soleus muscle fibers, and no synapses were formed. In the remaining 22 experiments, foreign axons successfully grew into the muscle in the immediate vicinity of the original endplates. In all 19 cases of this type that involved survival times greater than three weeks, the presence of foreign-innervated soleus muscle fibers was demonstrated either physiologically, by contractions evoked through the foreign nerve (18 muscles), or anatomically, by foreign synapses visible in silver-stained preparations (2 muscles). The total number of foreign-innervated soleus muscle fibers was generally quite small, however, ranging from just a few fibers to several percent ($\sim$100 fibers) of the whole muscle. This low degree of takeover was related mainly to the

limited extent to which the foreign nerve grew over the endplate region of the muscle. Within the region of overgrowth, the degree of take-over was much higher and sometimes involved a majority of the superficial muscle fibers that we sampled electrophysiologically.

Several results indicate that the foreign synaptic contacts were made directly at the original endplates, and not just in their general vicinity. No contractions upon foreign nerve stimulation were seen in soleus fibers whose endplates were outside the region of overgrowth of foreign axons (Fig. 1A). In silver-stained preparations intramuscular branches of foreign axons could be seen ramifying for several millimeters along the mid-portion of the muscle, but they made identifiable synapses only within the soleus endplate zone. In some cases careful inspection revealed single endplates that were supplied by both foreign and original nerves (Fig. 1B). Ectopic endplates were seen only rarely in cross-innervated muscles stained for acetylcholinesterase, and no examples of dual endplates were found in 32 fibers teased from the regions of cross-innervation in 6 muscles.

Intracellular recordings from single muscle fibers showed that about half of the foreign-innervated fibers retained their inputs from the original nerve (Fig. 2A). In some cases subthreshold endplate potentials (e.p.p.'s) were evoked by stimulation of one or both nerves, even though no neuromuscular blocking agents were present. When both inputs were subthreshold, the e.p.p.'s had similar time courses, but different amplitudes (Fig. 2B), providing further evidence that foreign and original synapses were in proximity. In the remainder of foreign-innervated fibers, however, stimulation of the original nerve evoked no visible response (Fig. 2C). Many of these recordings were

Fig. 1. Morphological evidence for foreign synapses at soleus endplates. A: Dark-field photograph of soleus muscle stained for acetylcholinesterase (AChE), and showing soleus nerve insertion (S), overgrowth of transplanted foreign nerve (F), and soleus endplate band (white dots). Bar with arrow underneath denotes the band of soleus muscle fibers in which the foreign nerve elicited contractions. This coincides with the region in which foreign axons grew in the immediate vicinity of soleus endplates. Occasionally, in other experiments, ectopic AChE was seen on a few fibers. In six cases, all from two muscles, these were actual ectopic endplates (perhaps resulting from injury to a few fibers, Miledi, 1963), but in all other cases the AChE was clearly non-synaptic and distributed like normal tendon AChE; teasing of single fibers indicated that the operation had induced bifurcations and/or abnormal terminations of fibers (Bormioli and Schiaffino, 1977). B and C: Silver-stained preparations from muscles in which the foreign nerve reached the soleus endplates. B: A foreign nerve branch (FNB) and an intramuscular branch of the soleus nerve (SNB) both innervate a single endplate (F+S). Also visible is another endplate innervated only by the soleus nerve (S). C: Several endplates are visible, two of which (S) are innervated only by the soleus nerve, and one of which (F) is innervated only by the foreign nerve.

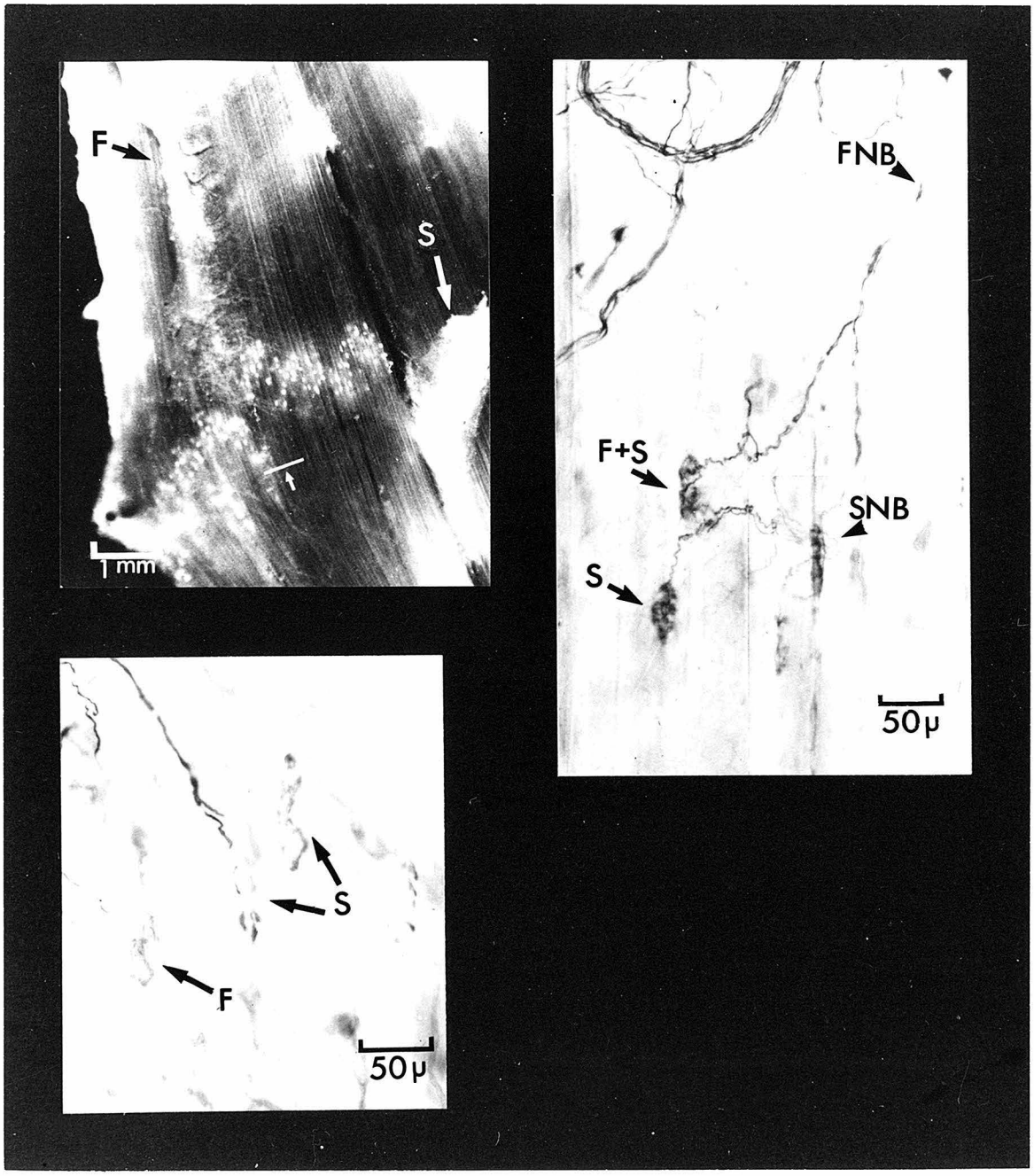
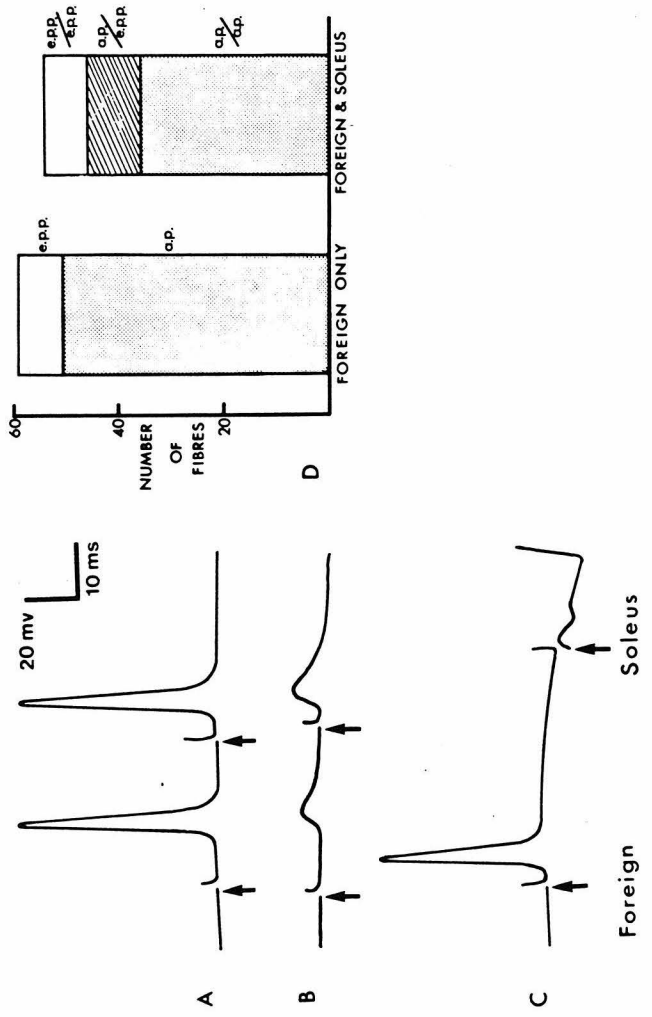

F
S
1 mm
FNB
F+S
SNB
S
50μ
S
F
50μ

Fig. 2. Physiology of foreign-innervated muscle fibers. A. Intracellular recording from a muscle fiber which responded with action potentials (a.p.'s) to stimulation of both foreign (first arrow) and soleus (second arrow) nerves. B: Recording from a fiber responding with an endplate potential (e.p.p.) to stimulation of either foreign or soleus nerves. C: Recording from a fiber which responded with an a.p. to foreign nerve stimulation and gave no response to stimulation of the soleus nerve. The field potential seen a few msec after second arrow was also recorded when the electrode was outside the muscle fiber. D: A bar histogram summarizing the physiological results. On the left are results from fibers innervated only by the foreign nerve, indicating the incidence of e.p.p. (open) vs. a.p. (stippled) responses to nerve stimulation. On the right are results for the dually-innervated fibers, showing the number of fibers giving a.p.'s to both nerves (stippled), e.p.p.'s to both nerves (open), or an a.p. to one with an e.p.p. to the other (hatched). For the 10 fibers showing mixed a.p./e.p.p. responses, 8 had e.p.p.'s to foreign nerve stimulation, and 2 to soleus nerve stimulation.


20 mv
10 ms
A
B
C
Foreign
Soleus
D
60
40
20
NUMBER OF FIBRES
e.p.p.
a.p.
FOREIGN ONLY
FOREIGN & SOLEUS
e.p.p./e.p.p.
a.p./e.p.p.
a.p./a.p.

sufficiently close to the endplate region that subthreshold responses, if present, would have been seen; their absence indicates that complete suppression of original synapses had occurred. Since we found examples in silver-stained preparations of endplates in the vicinity of the original nerve that were innervated only by the foreign nerve (Fig. 1C), it is likely that the suppression of original inputs involves the retraction and eventual disappearance of soleus nerve terminals. Fig. 2D shows the overall incidence of subthreshold vs. suprathreshold responses for the 59 fibers innervated only by the foreign nerve and for the 54 dually innervated fibers.

A comparison of results obtained at early (4 to 6.5 weeks) and late (7 to 14 weeks) survival times showed no large differences in the percentage of foreign-innervated fibers which retained inputs from the soleus nerve. Although this might reflect the early establishment of a stable outcome at each endplate, it seems more likely that the interaction between nerves is a dynamic process; thus endplates which are dually innervated at one stage might later become singly innervated, and endplates taken over completely by the foreign nerve might themselves be subject to a reestablishment of original inputs.

It was important to ascertain whether our surgical procedure led to any transient denervation or traumatization of synapses made by the soleus nerve that might have rendered muscle fibers abnormally susceptible to cross-innervation. Since denervated soleus muscle fibers are readily cross-innervated at ectopic sites (Frank et al., 1975), the absence of such ectopic endplates in almost all muscles argues against the occurrence of any substantial degree of denervation following surgery. Furthermore, in control experiments involving placement of a freshly excised

segment of the peroneal nerve over the soleus endplate region, no signs of denervation were seen in intracellular recordings from muscles examined at 1, 2, 3, 8, and 12 days after the operation. The morphology of endplates examined with silver staining 4, 5, and 16 days after such a dead nerve implant was also indistinguishable from normal. Thus, our particular surgical procedure apparently did not result in the markedly shrunken endplates and extensive terminal sprouting reported by others to occur in the vicinity of degenerating tissue (Jones and Tuffery, 1973). Additional evidence against a major role of nerve trauma came from control experiments in which the foreign nerve was crushed in the popliteal fossa either at the time of the transplant or in a separate operation two weeks later. Under these circumstances, foreign axons presumably did not reach soleus endplates until well after recovery from any trauma to the original nerve. Nonetheless, successful cross-innervation by the foreign nerve took place in all 5 experiments (out of 24 attempts) in which the foreign nerve grew into the endplate region of the muscle. No obvious differences were detected between these crushed-nerve transplants and our normal transplants in degree of cross-innervation. Moreover, the soleus nerve inputs were reduced or absent in 11 of 25 cross-innervated fibers sampled from these muscles; this incidence of suppression was not significantly different from that seen in our normal transplants ($p > 0.2$; χ^2 test).

The ability of foreign axons to suppress original nerve synapses may be related to a competitive advantage of terminals belonging to neurons with relatively few peripheral contacts (Brown et al., 1976; Purves, 1976a; Brown and Ironton, 1978). There is little if any advantage of appropriate vs. inappropriate skeletal motor neurons in the reinnervation of adult mammalian muscle (Purves, 1976b). Thus, in the present situation

foreign axons deprived of all their peripheral connections can compete successfully with and eventually displace soleus axons innervating their normal complement of muscle fibers, and their success might be related to this difference in number of pre-existing connections. In lower vertebrates, the interactions between foreign and original nerves are biased by relative affinities of motor nerves for appropriate muscles (Purves, 1976b), but here too the final outcome of any particular competitive situation may be affected by the overall peripheral fields controlled by different nerves.

Previous examples of synapse elimination in the adult nervous system have involved the loss of synapses which themselves were newly formed (after sprouting, Brown and Ironton, 1978; or regeneration, McArdle, 1975; Benoit and Changeux, 1978), and thus possibly immature. The present study suggests that mature neuromuscular synapses are also labile under appropriate circumstances, possibly even in completely normal muscles (Barker and Ip, 1966; but see Tuffery, 1971). If such lability exists for synapses in the central nervous system it could play a significant role in various types of normally and abnormally induced neural reorganization.

References - Chapter IV

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