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Formation and elimination of foreign synapses in salamander muscle

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FORMATION AND ELIMINATION OF FOREIGN SYNAPSES IN SALAMANDER MUSCLE

by

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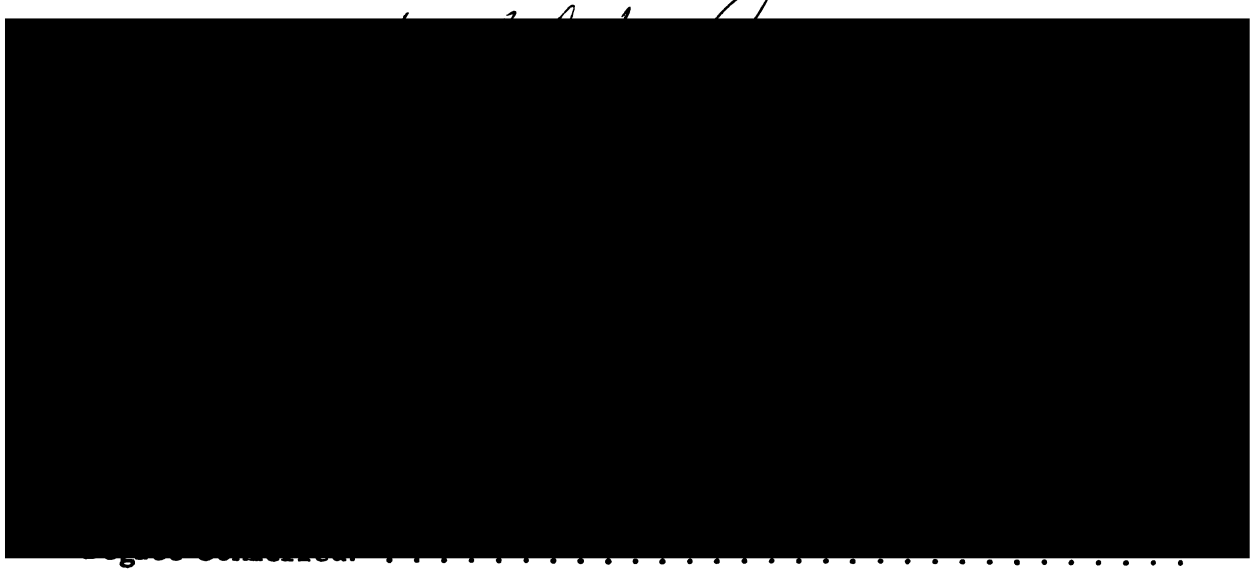

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ABSTRACT

Synapses by flexor nerve were induced on denervated extensor muscle in adult salamander forelimbs. Excitatory potentials evoked by these foreign synapses were at first small but increased in amplitude with time. Upon return of the correct nerve the efficacy of foreign transmission began to decline. The time of initiation of this decline was correlated with the resumption of correct nerve transmission. The suppression of foreign transmission involved a reduction in mean quantal content of transmitter release. Suppression of foreign synapses was sufficiently thorough that most ceased transmitting entirely. Both physiological and anatomical evidence indicate that suppressed foreign synapses are lost from the muscle.

Elimination of foreign synapses was investigated during salamander limb regeneration. Inappropriate neuromuscular synapses sometimes formed but were subsequently eliminated. Physiological distinction between the relative locations of correct and foreign synapses on single fibers revealed that correct and foreign terminals did not come into direct contact during suppression.

ACKNOWLEDGEMENT

To my parents, who gave me everything I have. And to Lucia, for her constant encouragement and patience. I am most grateful to Mike Dennis, a great mentor and friend. I thank Peter Sargent, Regis Kelly and John Heuser for their advice throughout this work. Thanks are also extended to Grete Fry and Nancy Johnson for their excellent technical assistance.

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Summary

1. Synapses by flexor nerve were induced on denervated extensor muscle in adult salamander forelimbs. Excitatory potentials evoked by these "foreign" synapses were at first small but increased to normal amplitude within several weeks, in the absence of correct nerve reinnervation.

2. Upon return of the correct nerve the efficacy of foreign synaptic transmission began to decline. The time of initiation of this decline correlated well with the resumption of correct nerve transmission. The suppression of foreign transmission involved a reduction in mean quantal content of transmitter release.

3. Suppression of foreign synapses was sufficiently thorough that most ceased transmitting entirely. Prior to reinnervation by the correct nerve 97% of the extensor muscle fibers received functional foreign synapses while four to six months after correct nerve return only 35% of the fibers retained foreign synapses, with weak transmission.

4. Two lines of evidence indicate that suppressed foreign synapses are lost from the muscle. (a) A second correct nerve lesion 6 to 8 months after the initial denervation produced no significant increase in the proportion of fibers with foreign transmission. (b) Four muscles which showed complete suppression of foreign transmission were bathed in medium containing horseradish peroxidase (HRP) and the correct nerve was stimulated repetitively. Subsequent histochemical staining for HRP and examination of synapses by electron microscopy revealed that 94% of the axon terminals had HRP incorporated into vesicles. Thus at least that percentage of all identifiable synapses were from the correct nerve.

5. During limb regeneration, inappropriate neuromuscular synapses

formed spontaneously but were subsequently eliminated.

6. The relative locations of correct and foreign synapses on regenerating muscle were determined by physiological criteria. It was concluded that foreign and correct terminals do not come into direct contact during suppression.

7. This ability to eliminate incorrect synapses in favor of correct ones is speculated to be a general characteristic of embryonic nervous systems, which in adulthood is retained by salamanders but lost by most other animals.

INTRODUCTION

Proper connections within the nervous system are very important from a functional point of view. How these correct connections originate during development is little understood, although considerable attention has been paid to the subject (for review see Sperry, 1951; Gaze, 1970; Jacobson 1970; Harris, 1974; Purves, 1976). There are two situations in which the formation of nerve connections can be studied, during embryogenesis and in adult nerve regeneration. The latter approach has received relatively more attention because of the technical difficulties encountered in experiments with embryos. Although these two processes of neuronal development are quite different, one hopes to find through study of adult nerve regeneration mechanisms involved in controlling the selective formation of nerve connections, which are common to embryogenesis.

The study of nerve-muscle interactions have and will provide much information on the development of the nervous system for the following reasons: 1) the nerve-muscle preparation is easily accessible so that hypotheses on formation of nerve connections may be tested using physiological, anatomical, biochemical and immunological techniques, and 2) peripheral nerves of vertebrates have the ability to regenerate and form functional connections again if their axons are severed.

Until recently the majority of the hypotheses on nerve-muscle interaction were proposed from behavioral observations of muscle function resulting after denervation and reinnervation, cross-innervation and limb transplantations, using particularly the lower vertebrates. The most notable of these hypotheses were Weiss' Resonance Theory, later modified to the Theory of Myotypic Modulation which became the predominant theory on the development of neuromuscular coordination for some forty years (for review see Weiss, 1936; 1941). In a series of careful experiments Weiss transplanted supernumerary limbs adjacent to the normal forelimbs

of larval and adult salamanders (Weiss, 1922; 1937a; 1937b; 1937c; 1937d). These implanted limbs were innervated by branches of the brachial nerves, which also innervated the adjacent normal limb. Upon activation of movement Weiss observed that the supernumerary limbs showed "homologous responses" to those of the normal limbs; that is, corresponding muscles in both the normal and the supernumerary limbs were excited synchronously. This situation was obtained regardless of the orientation of the implanted limb; thus, even when the dorsal ventral or anterior-posterior axis of the implant was reversed relative to the normal, the homologous muscles were still activated synchronously. On the basis of histological observations, Weiss (1937b) concluded that such homologous responses in an implant resulted if there was innervation by <u>any</u> of the brachial nerves.

In an effort to account for this apparent sufficiency of any brachial nerve to produce coordinated limb function, Weiss (1926) proposed a Resonance Principle of neuromuscular development. According to this scheme, the innervation of the developing limb was random, with each nerve to the limb sending branches to <u>all</u> of the muscles simultaneously. To explain the observed coordination of muscle activity, Weiss further proposed that each muscle responded only to a specific and characteristic pattern of nerve activity, that pattern being different for each muscle. Thus, he envisioned constant activity in the nerve with each muscle responding only when its particular pattern of activity appeared. This accounted for the homologous responses of implanted and normal limbs. However, this theory lost credibility when Wiersma (1931) recorded the activity of individual muscles and their nerves in the frog, and found that muscle contraction was always associated with impulse activity in the nerve, regardless of the pattern of that activity.

In a further effort to explain his behavioral observations, Weiss (1936) put forth a revised theory, that of Myotypic Modulation. His new postulate was that each muscle had its own specificity, which was intrinsic, and that this specific character "modulated"

the properties of the neurones which formed connections with the muscle. Thus, the central organization of a motoneurone was modulated by the muscle which it contacted so as to adapt to the correct function of that muscle. This theory had a profound impact on subsequent studies of the development of neuromuscular coordination, and it was used for the next thirty years to explain the recovery of function which follows peripheral nerve lesions in lower vertebrates. It did not, however, adequately explain all available experimental observations of nerve regeneration (Sperry, 1947; 1950; Sperry & Deupree, 1956; Arora & Sperry, 1957).

The theory of myotypic modulation remained in vogue until Sperry & Arora (1965) and Mark (1965) found that upon return of function in extraocular and fin muscles of fish following nerve lesions, the muscles were specifically reinnervated by their own nerves. In addition, when antagonistic muscles were carefully cross-innervated, uncoordinated use of the muscles was found to persist. These new findings ruled out the possibility that central organization of a motoneurone could be changed according to the function of its peripheral target. Recently Grimm (1971) cross-innervated flexor and extensor muscles of the axolotl forelimb and confirmed that functional recovery of the muscles was due to selective reinnervations of the muscles by their original nerves. Landmesser & Morris (1975) reported that the pattern of innervation of hind limb musculatures in the chick embryo, as determined by visual contraction and muscle tension recorded during stimulation of specific spinal roots, was similar to the adult the earliest time that muscle function could be demonstrated. They concluded that chick motoneurones were specified with respect to their peripheral destination and grew out selectively to synapse with appropriate muscles from the onset. It is not clear, however, whether minor inaccuracies in the initial innervation pattern would have been revealed by this technique.

Another situation where selectivity in neuromuscular innervation was indicated was in the slow and fast muscles of amphibia and rats. These two types of muscles differ in their

physiological properties and mode of innervation (Kuffler & Vaughan Williams, 1953; Close, 1964). In the frog some muscles such as the pyriformis muscle, contain both fast and slow fibers. When these are denervated the slow muscle fibers are initially innervated by fast nerves (Miledi & Orkand, 1966) but this innervation is subsequently replaced with the appropriate slow innervation (Elul et al., 1970; Schmidt & Stefani, 1976). In both the toad and the rat, Hoh (1971, 1975) demonstrated that fast and slow muscles show strong preferences for correct synaptic connections (i.e. fast nerve to fast muscle and vice versa) under situations of competitive reinnervation by both fast and slow nerves.

Thus, the myotypic modulation hypothesis has been contradicted by considerable evidence that selective reinnervation does occur. In retrospect it is apparent that (a) Weiss' behavioral observations were not sufficiently sensitive to distinguish whether there was appropriate use of all of the individual muscles in supernumerary limbs upon innervation by branches of the brachial nerve, and (b) he was incorrect in assuming that implantation of a single brachial nerve into a supernumerary limb necessarily prevented access of the normal variety of motor nerves to that limb (Weiss 1937b).

It is of interest to know how specific nerve connections are formed during development. One possibility is that there is a one to one specificity in synapse formation between a neurone and its target, such that a neurone would synapse only with its appropriate target from the beginning. This is certainly not the case in neuromuscular connections. Mammalian skeletal muscle fibers are polyneuronally innervated at birth, yet all but one of the synapses are physically eliminated within the first two weeks (Redfern, 1960; Bagust, Lewis & Westerman, 1973; Brown, Jansen & Van Essen, 1976; Rosenthal & Taraskavich, 1977). The mechanisms underlying this synapse elimination are not known. Nevertheless it indicates that a one to one specificity from the beginning in the formation of neuromuscular synapses is not the rule.

Attempts to demonstrate selective reinnervation of adult

mammalian skeletal muscles by their appropriate motoneurones have been mostly unsuccessful. In every case where incorrect innervation has been induced (Weiss & Hoag, 1946; Bernstein & Guth, 1961; Tonge, 1974; Frank, Jansen, Lømo & Westgaard, 1975; Brown, Jansen & Van Essen, 1976), transmission from these foreign nerves persists upon reinnervation by the correct nerve. This lack of selective reinnervation in adult mammalian skeletal muscles was consistent with earlier behavioral observations that coordinated use of reinnervated muscles was lost (Sperry, 1945). Although inappropriate synapse elimination does not occur in adult mammalian skeletal muscles, it is possible that the phenomenon may be involved in the formation of specific nerve connections in the embryo but lost in adulthood.

One form of suppression of inappropriate synapses was proposed by Mark and his collaborators (Marotte & Mark, 1970a; Mark & Marotte, 1972). Their principle experiment was to transplant a nerve from an antagonistic muscle onto a denervated extraocular muscle of the carp and then to monitor the optokinetic reflexes in response to body tilt. The first behavioral response to tilting up and down the head of the fish appeared after a delay of some weeks and was inappropriate, as might be expected if successful cross-innervation had occured. But when the original nerve was allowed to grow back to the muscle, the optokinetic reflex eventually returned to normal. From these behavioral observations, they concluded that the function of foreign synapses was suppressed upon reinnervation by the correct nerve. The absence of degenerating nerve terminals during the "take over" of the muscle by the correct nerve led them to propose further that suppressed foreign synapses remained morphologically normal, although silent (Marotte & Mark, 1970b; Mark et al., 1972). Central reorganization of motoneurones was rejected by these investigators (Mark & Marotte, 1972) as an alternative explanation for the return of the correct reflexes on the basis that while action potentials were recorded from foreign axons, no muscle contraction in response to these nerve impulses was observed.

One major weakness in their experimental design was that subthreshold muscle responses would not have been detected. Thus, some function of foreign synapses may have remained. Moreover, changes in the efficacy of synaptic transmission and of transmitter sensitivity beneath the foreign nerve terminals after correct reinnervation which provide alternate explanations for the loss of foreign nerve functions were not excluded by Mark's behavioral experiments.

Despite dubious evidence, Mark's hypothesis on synapse suppression aroused considerable interest. Such suppression of inappropriate synapse might represent one of the mechanisms which give rise to the high degree of specificity that exists in the structure of the mature nervous system. In addition, if synapses could be turned on and off, this phenomenon of synapse suppression might form the basis of cellular learning and memory (Mark, 1974). More important, the proposal of silent synapses implied that millions of synapses within the central nervous system might be nonfunctional and consequently the traditional anatomical criterion of defining functional pathways by the presence of vesicle filled nerve terminals would require immediate reinvestigation. The impact of Mark's proposal of silent synapses has been profound. Unfortunately the evidence leading to the proposal of his hypothesis was insufficient and ambiguous.

Scott (1975) repeated the experimental procedure followed by Mark and observed the same change in pattern of the extraocular reflexes. However, she observed that even after return of the appropriate reflex, the muscle still contracted in response to tetanic stimulation of the foreign nerve. In addition, she was able to record foreign synaptic potentials after the muscle had been reinnervated by its correct nerve. For these reasons, Scott came to the conclusion that suppression of foreign synapses did not occur in the carp eye muscles as had been proposed by Mark. She believed, instead, that the loss of the inappropriate reflex resulted from regeneration of the excised muscle. Unfortunately, Scott's work suffers from some of the same weakness as Mark's --

namely that she did not determine the efficacy of foreign synaptic transmission as a function of time after correct reinnervation. It is conceivable, and not excluded by her work, that the amount of transmitter released from foreign terminals does decline even if it is not completely eliminated; this would still constitute suppression of function. Thus, a definitive statement about the presence or absence of foreign synapse suppression in goldfish extraocular muscle awaits further electrophysiological examination. Such an examination has been conducted in another fish muscle; Frank and Jansen (1976) experimentally induced and studied the fate of foreign synapses on gill muscles of the perch. In that system reinnervation by the correct nerve caused no change in efficacy of transmission by foreign nerve.

In amphibia several studies, including that of Grimm discussed above, indicated a selective preference for correct reinnervation of muscle fibers. Cass, Sutton and Mark (1973) examined the competition between correct and foreign nerves for reinnervation of hindleg muscle in axoltol, using primarily evoked twitch as a monitor of the state of innervation. Following section of one of the spinal roots innervating the hind limb, the territories of adjacent roots spread to take over denervated muscle fibers, presumably by collateral sprouting. But when the original nerve regenerated, the original pattern of innervation was restored. Furthermore if the previously severed root was cut a second time, then establishment of functional synapses by the adjacent nerve roots took three days instead of three weeks as after the first interuption. These results led them to conclude that foreign synapses were suppressed, but remained present in the muscle after it was reinnervated by its correct nerve.

Fangboner and Vanable (1974) denervated the superior oblique muscle of Xenopus larvae and from histology combined with observation of evoked contraction showed that it was spontaneously reinnervated by both its correct (IV cranial) nerve and fiber from the IIIrd cranial nerve (incorrect). These incorrect nerve sprouts were subsequently withdrawn in a manner which suggested that

competition with the correct nerve was responsible for their displacement. The fact that the number of incorrect (N III) sprouts in the muscle declined with time, was a strong indication against retention of silent synapses after their suppression, as had been proposed by Mark and associates.

Given the significance of such a process of synapse suppression and the lack of relevant physiological information at the cellular level I decided to reexamine foreign-correct synapse competition in salamanders in more detail, making use of both intracellular recording and selective labelling of synapses for electronmicroscopy.

My approach to this problem has been to induce foreign innervation on an extensor muscle of adult salamander and then follow in time the properties of such "foreign" synapses consequent to correct reinnervation. Whether there is loss of function of foreign synapses can be determined by stimulating the foreign nerve while recording intracellularly from single muscle fibers. If foreign transmission persists after correct reinnervation and incorrect use of the muscle is the result, then suppression of foreign synapses does not occur. On the other hand if foreign transmission persists and there is correct use of the muscle, then central reorganization may be indicated. In such a situation one needs to be careful to determine that correct axons have not reinnervated their proper targets by way of the foreign nerve trunk.

Assuming that there is loss of function of foreign terminals as a result of correct reinnervation one can ask whether this suppression of function involves one or more of the following mechanisms: 1) A decline in the quantal content of transmitter released; 2) A change in the transmitter sensitivity beneath the nerve terminals, and 3) Physical elimination of foreign nerve terminals. The combined use of intracellular recording and electronmicroscopy should distinguish which of these mechanisms is involved in synapse suppression. Should synapse suppression result from a decrease in post-synaptic sensitivity to transmitter at foreign nerve terminals, then the miniature endplate potentials

recorded while suppression is in progress ought to be smaller than normal. In such a situation one would expect to record small minepps arising from foreign terminal release as well as normal amplitude minepps from correct nerve terminals, producing an amplitude distribution which is skewed instead of normal. Such a skewed distribution of miniature endplate potentials would also result if suppression involved a decline in the number of transmitter molecules per quantal packet released from foreign terminals with no change in the post-synaptic sensitivity. Still another possible cause of foreign suppression is a decline in the quantal content of evoked transmitter release from foreign terminals. The quantal content of transmitter release can be investigated physiologically (cf. del Castillo and Katz, 1954).

The fate of suppressed synapses, whether they are morphologically normal but silent, as Mark has claimed, or physically eliminated from the muscle, can be resolved by electronmicroscopy using selective labels for correct and/or foreign nerve terminals. The nature of correct and foreign nerve interactions which leads to a suppression of function at foreign synapses may also be studied by both intracellular recording and electronmicroscopy.

I have also studied "suppression of foreign transmission" in a developmental situation, that of limb regeneration in adult salamander. The possibility of spontaneous formation and subsequent elimination of inappropriate nerve-muscle synapses was investigated in two adjacent antagonistic forelimb muscles. The demonstration of foreign synapse suppression in both experimentally cross-innervated adult and in the regenerating limb of salamanders, described below, raises the possibility that foreign synapse suppression may be one of the mechanisms involved in the formation of proper nerve connections during development.

METHODS

A. Adult Cross-innervation

Surgical Procedures. Adult newts, Notopthalmus viridescens, were anaesthetized with tricaine (1/1000 in Ringer solution). The forelimb flexor (humero-antibrachialis) muscle was excised and its nerve displaced laterally to the surface of the extensor (anconeus) muscle. The animals were returned to tanks of tap water and fed weekly with brine shrimp. Two weeks after the initial operation, the correct nerve to the anconeus muscle was crushed in one series of animals and resected in another. In some of these animals, the correct nerve was crushed or resected a second time 6 to 8 months after the initial nerve lesion.

Physiological Recordings. At various times after the correct nerve lesion, the animal was pithed and the entire limb was removed with the 3rd, 4th and 5th nerves. The spinal nerves were dissected through the inferior and superior brachial nerves, which innervate respectively flexor and extensor musculature in the upper arm (see Grimm, 1971, for further description of innervation pattern). The limb was skinned, pinned on a Sylgard dish with a glass coverslip bottom and the trunks of the two nerves were drawn into separate suction electrodes. All experiments were performed at room temperature in normal frog Ringer of the following composition (in m-moles/l): NaCl, 120; KCl, 2; $CaCl_2$, 1.8; glucose, 10; HEPES buffer, 4 (pH 7.2).

Intracellular recordings were made using glass micropipettes filled with 4M K-acetate and having resistances of 50-100 Mohms. The signals were fed through W.P. Instruments M4A amplifiers, 1X gain, and into a Tektronix D11 storage oscilloscope.

The quantal content of foreign nerve transmission was estimated both by the method of failures and from the coefficient of variation of respones (cf. del Castillo & Katz, 1954) evoked by stimulating

200 or more times at a frequency of 0.2 sec^{-1}. There was no significant difference between the values determined for individual fibers by these two methods.

Histological Techniques. Active nerve terminals were labelled by stimulating uptake of horseradish peroxidase (HRP) into synaptic vesicles (Heuser & Reese, 1973). The muscle was pinned on Sylgard in 0.5 ml chamber and presoaked for an hour in 10 mg/ml HRP (Worthington) in normal frog Ringer. Equilibration of HRP into the extracellular space was facilitated by washing the solution back and forth over the muscle with a Pasteur pipette. The nerve of interest was stimulated for 7 minutes with 10 Hz trains of 1 s duration alternating with 1 s rest. The preparation was then allowed to rest for 3 minutes. This pattern of intermittent stimulation and rest was repeated three times and the muscle allowed to rest in HRP for an hour. The muscle then was extensively washed with frog Ringer containing 0 Ca^{++}, 10 mM $MgCl_2$ and 0.5 mM EGTA. Solutions were changed throughout the wash. The muscle was fixed for 2 hr in 2% paraformaldehyde and 1% glutaraldehyde with 30 mM HEPES (pH 7.2), 50 mM NaCl and 5 mM $CaCl_2$, left overnight in the same HEPES-buffered salt solution, and then chopped into narrow bundles of muscle fibers with a Smith-Farquhar tissue chopper. These bundles were reacted histochemically with hydrogen peroxide and diaminobenzidine according to the method of Graham & Karnovsky (1966), post-fixed with osmium, embedded in araldite, and thin sections of synapses were obtained randomly throughout the muscles.

B. Limb Regeneration

Both forelimbs of adult newts were amputated under ether anaesthesia (1% in water). They were returned to their tanks, fed and awaited for limb regeneration at mid and late digit stages (Iten & Bryant, 1973; Dennis, 1975). About 20 flexor muscle fibers (humero-antibrachialis) in each limb were recorded from, proceeding from the edge adjacent to the extensor muscle (anconeus) while

independently stimulating the flexor and extensor nerves (see above).

Relative Locations of Correct and Foreign Synapses. The relative locations of correct and foreign synapses were determined physiologically in the following manner. Fibers innervated by both correct and foreign nerves were identified by independently stimulating each nerve. A hypertonic sucrose solution (0.5 M) was applied focally by pressure injection from a blunt pipette that could be placed at different sites along the muscle fiber. An increase in the frequency of spontaneous endplate potentials during injection would indicate that a terminal was present underneath the pipette (cf. Fatt & Katz, 1951). The medium was then replaced with a Ca^{++} free Ringer (0.5 mM EGTA). A 0.5 M Ca^{++} solution was then applied focally again by pressure at the predetermined synaptic site, while simultaneously stimulating the correct and foreign nerves. Simultaneous onsets of both correct and foreign responses during Ca^{++} application would indicate the presence of correct and foreign terminals at the same synaptic site.

RESULTS

Properties of Normal Anconeus Fibers. Muscle fibers varied from 2 to 4 mm in length and 20-30 μm in diameter. The resting potentials were in the range of -90 to -110 mV. Spontaneous and evoked end-plate potentials could be recorded anywhere along the muscle fiber (see also Lehouelleur & Chatelain, 1974 and Dennis, 1975). There was no evidence of electrical coupling between muscle fibers (Dennis, 1975).

To check for multiple innervation, the intensity of stimulus impulses to the nerve was varied in small steps from below threshold to supramaximal. In 120 fibers sampled (8 muscles), 24 had a single synaptic input, 60 had two inputs and 36 received three inputs. Cholinesterase staining revealed that synapses were distributed at several sites along the length of individual fibers.

Muscles were checked for inappropriate synaptic inputs by independently stimulating the superior and inferior brachial nerves while recording from single fibers. Of 250 fibers sampled in 9 muscles, none received foreign synaptic input.

Innervation of Fibers by Implanted Foreign Nerve. About 2 weeks after lesion of the correct nerve, foreign transmission began to appear in some muscle fibers. At this time repeated stimulation of the foreign nerve evoked end-plate potentials of low and variable amplitude, often with intermittent response failures. At longer times after correct nerve lesion the population of fibers innervated by the foreign nerve increased and was virtually complete within one month, provided the correct nerve was kept away. The foreign synaptic responses also increased in magnitude with time, and came to resemble those seen in normally innervated adult fibers; some muscle fibers were multiply innervated by foreign axons, as indicated by distinct inputs of differing thresholds (Fig. 1) and in some the end-plate response was large enough to cause an action potential and twitch.

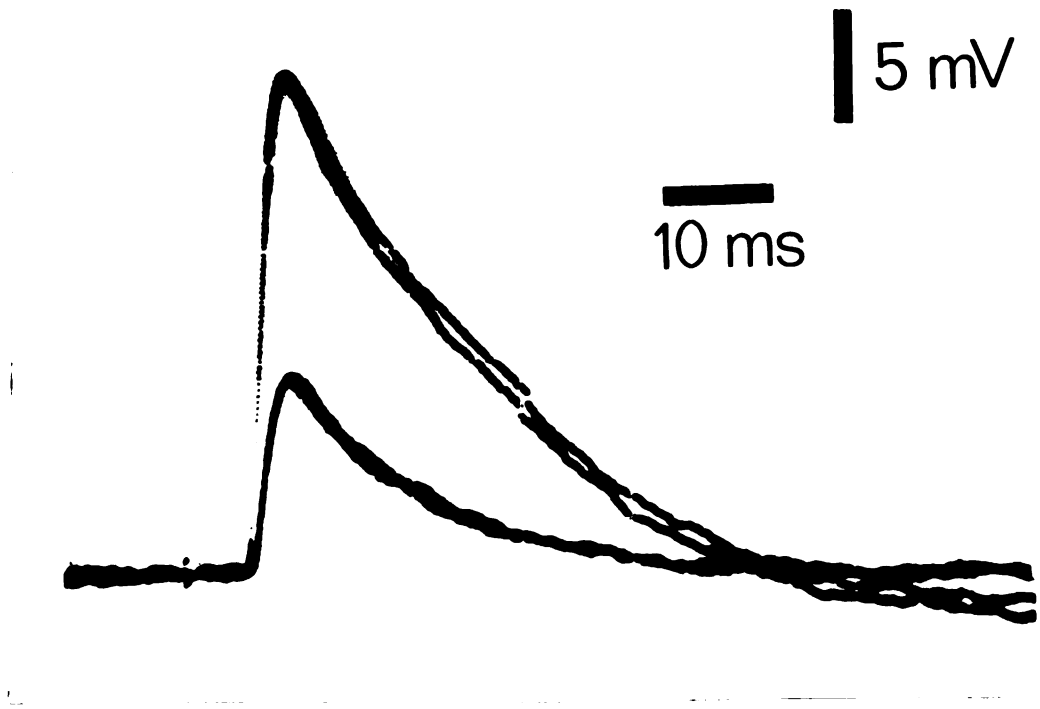


Figure 1

Foreign synaptic responses 45 days after correct nerve resection. The fiber was innervated by two motoneurones as revealed by the increase in response amplitude with increase in the intensity of stimulus impulses to the nerve. Two responses were recorded at each stimulus intensity. No response was obtained from stimulation of the correct nerve.

Decline in Quantal Content of Foreign Transmission. Approximately 2-4 weeks after the correct nerve was crushed or 6-8 weeks after it was resected, it began to reinnervate the muscle. The reformed correct synapses rapidly returned to their normal state of suprathreshold transmission (Fig. 2A). Conversely, foreign responses evoked subsequent to correct reinnervation were often of low amplitude (Fig. 2B) and failures were again frequent. In fibers in which foreign nerve stimulation was so associated with intermittent release failures, the amplitudes of the evoked foreign responses were distributed as predicted by the failures method, using Poisson statistics (cf. del Castillo & Katz, 1954). Furthermore, when the amplitude histogram of these evoked foreign responses was compared to that of the miniature end-plate potentials (min.e.p.p.s) recorded at the same locus, the mean amplitudes of the spontaneous and the unitary evoked events were identical (Fig. 3), and were within the normal range (0.5 - 2.0 mV). Presumably the min.e.p.p.s in such dually innervated fibers arose from transmitter release by correct as well as foreign terminals. Both the Poisson nature of foreign release and the identity in mean amplitude of spontaneous and foreign-evoked potentials indicate that the small foreign responses seen after correct reinnervation result from a low quantal content, rather than a reduction of quantal unit size or of transmitter sensitivity beneath foreign foreign terminals.

The quantal content of foreign transmission was estimated in individual fibers at various times after the correct nerve lesion. In both series of animals studied, correct nerve resected and correct nerve crushed, the mean quantal content of foreign transmission declined with time, as illustrated in Fig. 4. The initiation of this decline was more rapid following nerve crush than after nerve resection, and was in both situations coincident with the onset of reinnervation by the correct nerve. This suggests that correct reinnervation and foreign suppression are causally related. It should be noted that the data which were used in deriving the mean quantal content at each time point

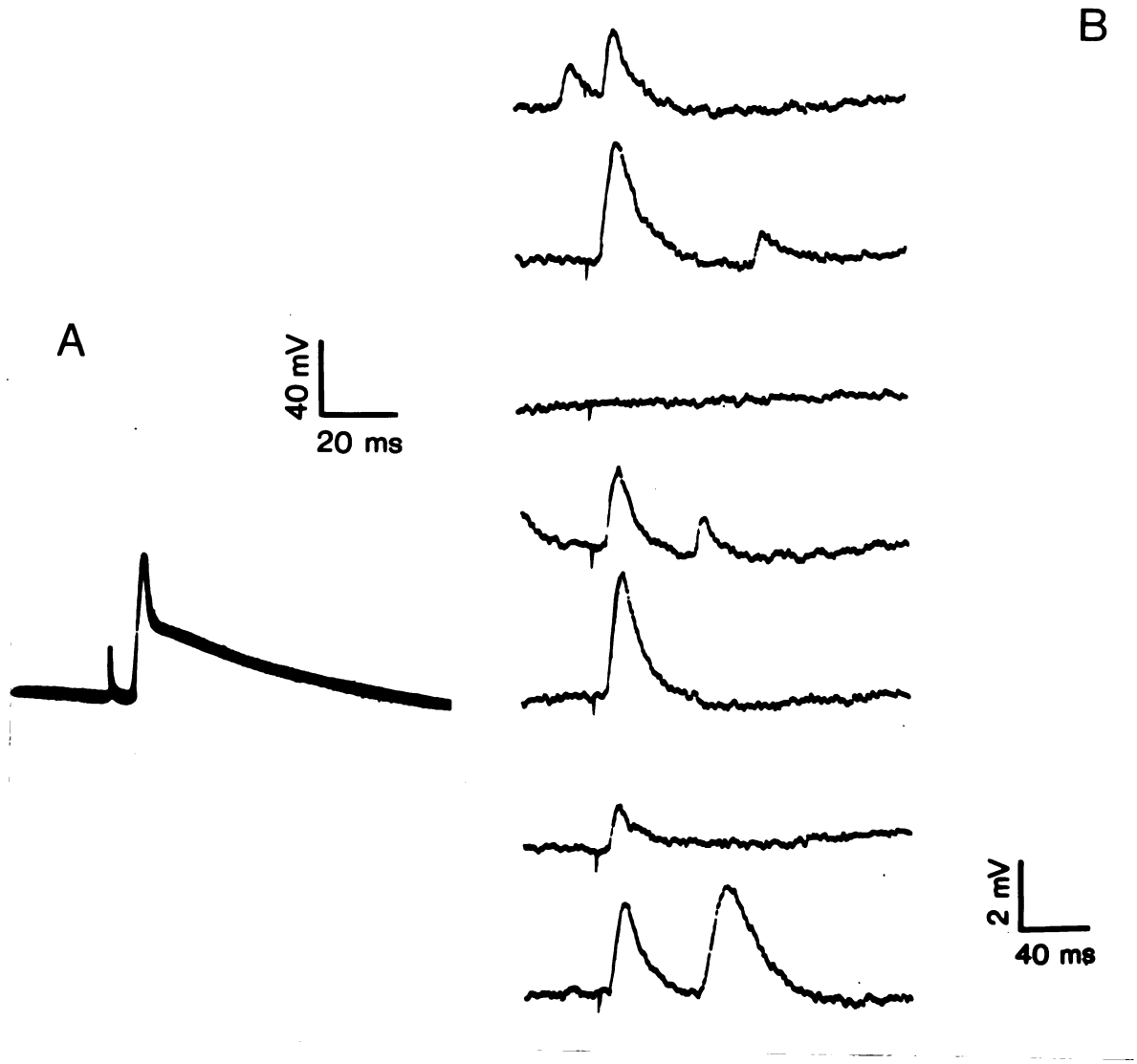


Figure 2

Recordings from a single fiber dually innervated by the correct and foreign nerves, 57 days after the correct nerve resection. A, large end-plate response with action potential superimposed evoked by correct nerve stimulation. B, consecutive responses to repeated maximal stimulation of the foreign nerve at a frequency of 1 sec^{-1}. A nerve stimulus of constant intensity occurs near the beginning of each sweep, as indicated by the artefact. With some stimuli, the foreign nerve failed to release transmitter (trace 3 from top). Spontaneous potentials appear randomly in several of the traces (1, 2, 4 and 7 from top).

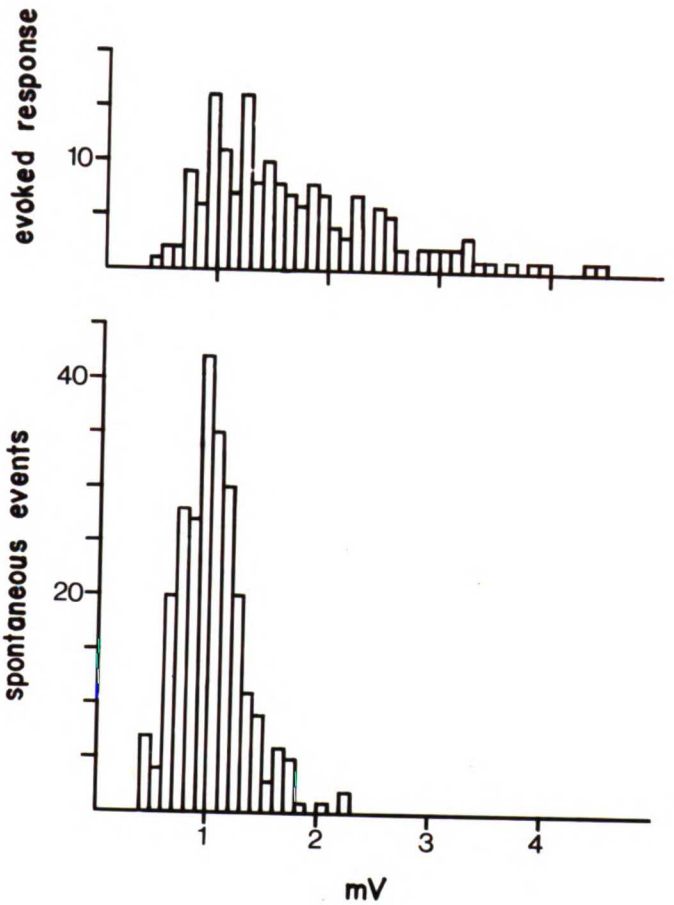


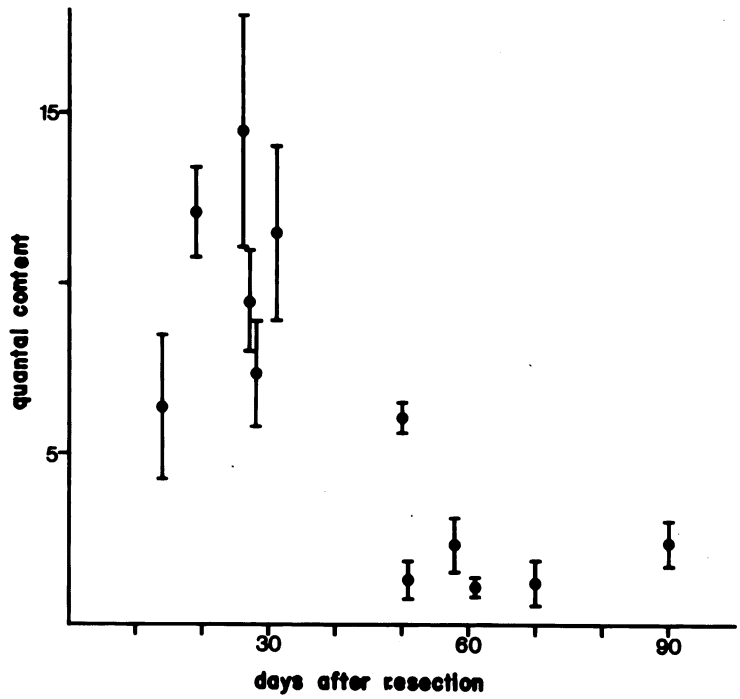
Figure 3

Amplitude histograms of spontaneous (bottom) and foreign evoked (top) responses recorded from a fiber 50 days after correct nerve resection. The foreign nerve was stimulated 294 times with 114 failures. The mean amplitude of the foreign evoked responses = 1.05 mV while that of the spontaneous events = 1.08 mV. Stimulation of the correct nerve resulted in an action potential. Medium contained 1.8 mM calcium.

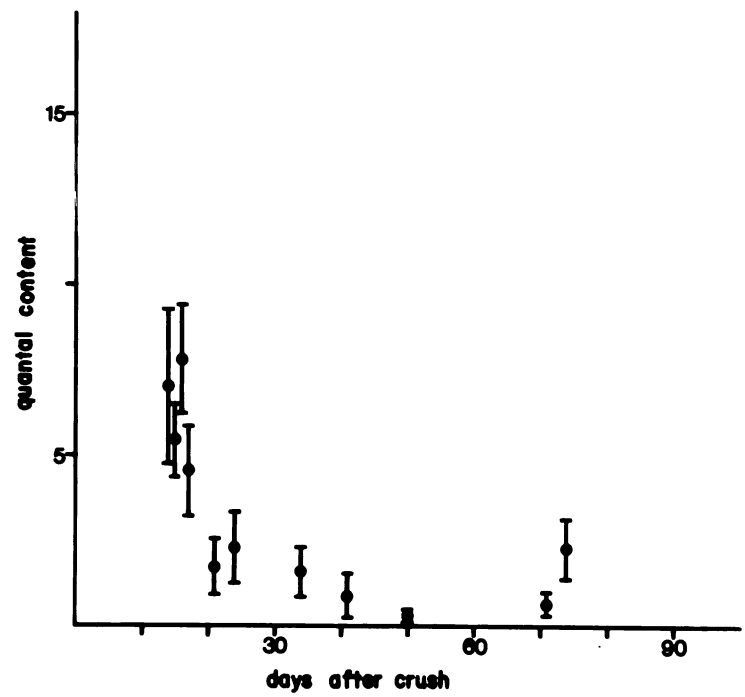
Figure 4

Mean quantal content of foreign nerve release plotted as a function of time after the correct nerve was resected (A) and crushed (B). Each point is the average of the quantal contents measured in 6 - 10 fibers. Each vertical bar represents the standard error of the mean. Fibers which showed no foreign responses were not used in calculating the mean. Note that the quantal content of foreign transmission becomes higher and remains so longer when the correct nerve is resected.

A


15
5
quantal content
30
60
90
days after resection

B


15
5
quantal content
30
60
90
days after crush

in Fig. 4 were taken only from fibers which did show some foreign response. That is, fibers which showed no foreign response (m = 0) were not used in calculating the mean. Thus, these figures underestimate the decline in foreign synaptic transmission with time (see below).

In two muscles where correct reinnervation by chance failed to occur or was sparse, the extent and efficacy of foreign transmission remained high, even 9 months after correct nerve lesion. In both cases the foreign nerve had reinnervated its normal target (flexor) musculature as well.

Decline in Extent of Foreign Transmission. To get another index of the suppression of foreign synapses, the extent of functional foreign innervation upon correct reinnervation was investigated. At various times after the correct nerve resection, intracellular recordings were made from single muscle fibers and the correct and foreign nerves independently stimulated. In 10 muscles examined 12 to 47 days after resection, 97.3 $\pm$ 1.5% of 215 fibers received foreign input, wheras in 29 muscles 57 to 299 days after resection only 269 of 673 fibers (39.5 $\pm$ 6.5%) had functional foreign input. Thus the extent as well as the efficacy of foreign transmission declined upon reinnervation by the correct nerve, even though the suppression was not always complete.

Second Resection of Correct Nerve 6 to 8 Months after the First.

In light of earlier claims of morphological integrity of suppressed foreign synapses (Mark et al., 1972; Cass et al., 1973) it was of interest to know the fate of such synapses in this system. To determine whether suppressed foreign synapses could be rapidly reactivated upon loss of correct innervation the correct nerve was resected a second time long after the correct nerve had regenerated (6 - 8 months after the first resection). In 17 muscles (383 fibers) sampled 2 - 20 days after a second correct nerve resection, an average of 36.2 $\pm$ 9.8% of the fibers had functional foreign inputs. By comparison, in 17 control muscles (453 fibers), of the same experimental age whose correct nerve had been resected only once 6 - 8 months earlier, 35.1 $\pm$ 7.8% of the fibers still had

some functional foreign innervation. The large standard error of these values reflects the considerable variability encountered from muscle to muscle; in some, no foreign transmission could be detected. The end-plate responses to foreign nerve stimulation were similar in both sets of animals, typically of 1 to several mV amplitude. Thus, foreign transmission did not increase in extent or efficacy, up to 20 days after a second correct nerve resection, at long times after correct reinnervation. Since the initial development of foreign transmission took place in 14 - 18 days (Fig. 4), these results suggest that foreign synapses are not available for reactivation at long times after their suppression.

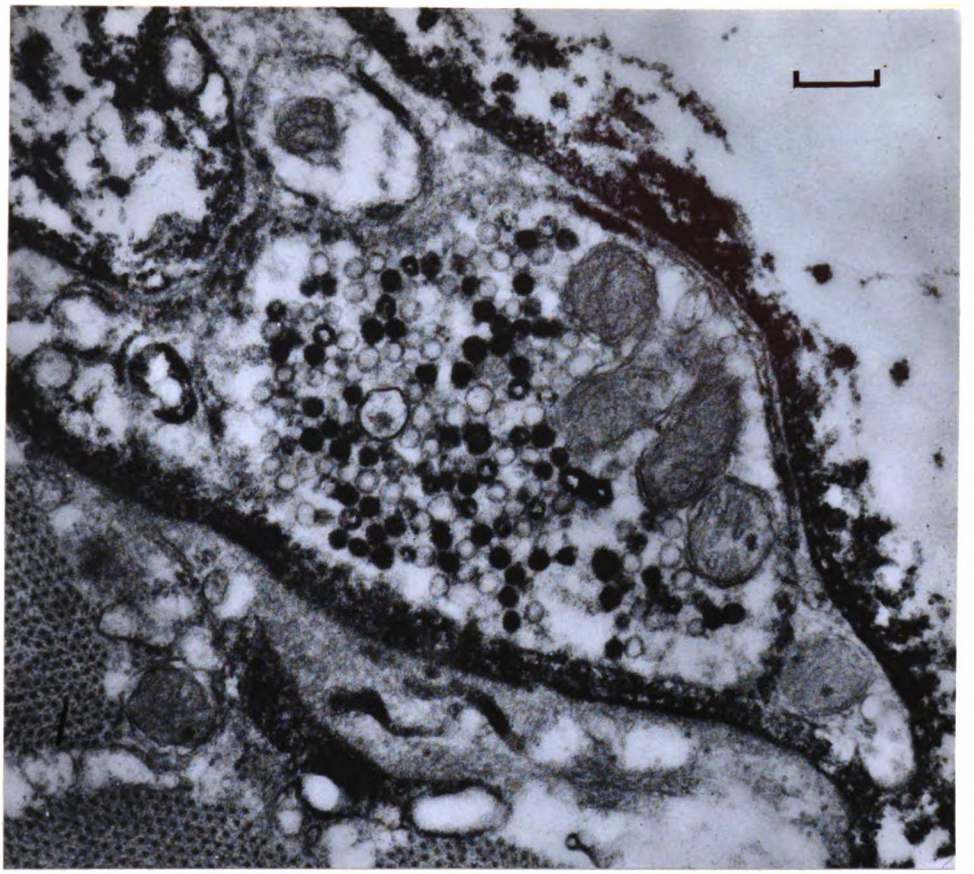
HRP Labelling of Active Nerve Terminals. The best evidence for the existence of morphologically normal but functionless synapses would be a demonstration of their presence by electron-microscopy. For this purpose, one would like to be able to distinguish correct from foreign synapses. Cutting the correct nerve so as to use its degeneration products to distinguish it from foreign terminals (Mark et al., 1972) is not satisfactory for the following reasons: 1) Some synapses may be degenerating more slowly than others. 2) Some of the normal looking synapses may have been newly formed by sprouting of foreign axons subsequent to recutting the correct nerve and could be functional, rather than silent. 3) Some of them may have been subthreshold functional foreign synapses not detected by the test criteria used. Therefore I used a different approach to study the morphology of suppressed foreign synapses. I made use of the observation of Heuser & Reese (1973) that horseradish peroxidase (HRP) is taken up by active nerve terminals during vesicle recycling to label correct nerve terminals. My intention was to so mark correct terminals and thus distinguish the foreign as the unlabelled fraction of the population.

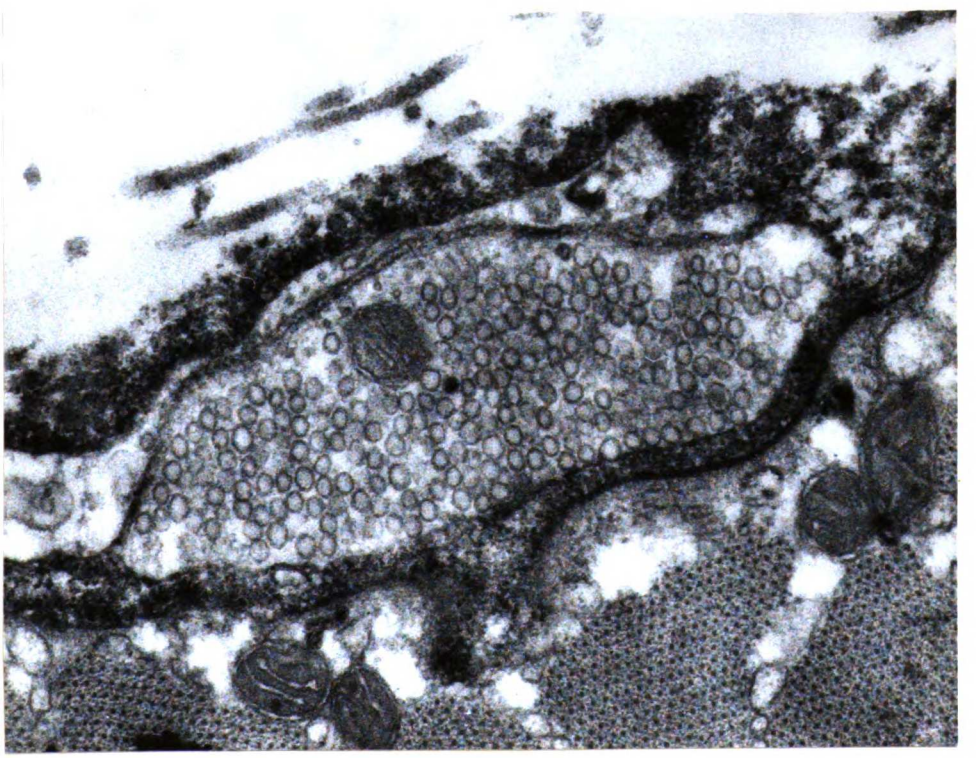
To make sure that the technique was applicable in this system, two normal muscles were prepared for HRP experiments, and the correct nerve stimulated as described in Methods. In 61 end-plates studied, 95% were positively labelled as judged by the presence of HRP reaction product on their vesicles. In many of these terminals, more than 50% of the vesicles were labelled (Fig. 5A). This was

Figure 5

(A) Cross section of a normal anconeus end-plate stimulated for HRP uptake as described in text. The nerve terminals, surrounded by a thin layer of Schwann cell cytoplasm, contains synaptic vesicles and a few mitochondria. 48% of the vesicles contain the electron dense peroxidase reaction product, indicating they contain HRP.

(B) Cross section of a normal anconeus terminal soaked in HRP but not stimulated, to show background uptake. Calibration bar, 2 μm.





clearly different from background uptake (Fig. 5B), determined in another muscle which was soaked in HRP but not stimulated; here only one or a few vesicles per terminal were labelled.

Normal nerve terminals were characterized by an abundance of clear vesicles, a few with dense cores, mitochondria, occasional neurofilaments, and a thin layer of Schwann cell cytoplasm over their outer surfaces. Terminals were closely apposed to muscle membrane which in some synapses showed well established subsynaptic folds. Their ultrastructure was similar to that reported by Lentz (1969) for the mature newt synapse. Most of the synapses showed only a single nerve ending. Occasionally two or three axon terminals were found apposing a single end-plate, but it was not certain whether these were branches of the same axon or were terminals from different motoneurones.

Four cross-innervated muscles which showed no sign of remaining foreign transmission by intracellular recording (125 to 274 days after correct resection) were prepared for HRP uptake. After correct nerve stimulation 94 $\pm$ 3% of 293 terminals showed positive HRP labelling of their vesicles. To make sure that foreign terminals located in a restricted region of the muscle were not missed by selective sampling, samples of synapses were taken randomly throughout the muscle; in one case, the long axis of a muscle was cut into 3 pieces, each was re-embedded and samples taken from each segment. This extent of labelling is like that observed in control experiments on normal muscles (95%) and suggests that few if any of the synapses on these muscles are foreign. In the absence of physiological signs of foreign innervation following correct reinnervation, foreign terminals are not physically present.

HRP uptake in two muscles which did not show complete suppression of foreign transmission was also studied, 225 and 299 days after correct nerve resection. In these muscles 30% of the fibers sampled had residual foreign transmission. Upon correct nerve stimulation in HRP, 68% of 150 terminals were labelled, 32% were not. There was no obvious difference in ultrstructure between labelled and unlabelled terminals.

In one muscle, which by intracellular recording showed no sign of foreign response, 228 days after the initial correct resection the foreign nerve was stimulated in the presence of HRP. Of 27 synapses examined all showed no more than background uptake. This negative result served as an additional control against background uptake by unstimulated (in this case, correct) terminals and further supported the conclusion that suppressed foreign terminals are eliminated.

It was of interest to know whether suppression of foreign transmission required direct contact between correct and foreign terminals, especially during the early stages of correct reinnervation when profiles of several terminals are often seen at single end-plates. With this in mind, HRP labelling of the correct nerve was attempted in several muscles at a time when correct reinnervation was in progress. Some of the terminals were labelled and others were not. However, it was not certain that all of these unlabelled terminals were foreign since some of the newly developing correct synapses would be expected not to transmit (Dennis, 1975) due to failure of action potentials to propagate into their terminals (Dennis & Miledi, 1974). Such terminals would not take up HRP. To check this possibility I simply denervated and awaited reinnervation of an anconeus muscle, without introducing a foreign nerve. At the time of ongoing reinnervation, the muscle was soaked in HRP and its nerve stimulated as above. Upon examination in EM multiple axon profiles at single end-plates were found with some terminals labelled while others were not. Thus, the HRP labelling technique did not permit us to unequivocally distinguish correct from foreign terminals at the time when correct reinnervation was in progress.

Elimination of Inappropriate Synapses during Salamander Limb Regeneration. One way to find out if suppression of foreign transmission is involved with formation of proper nerve connections is to ask whether mistakes in nerve connections are made in a developmental situation and if so whether such connections are corrected. Mistakes in nerve connections were sought by recording intracellularly from an immature forelimb flexor muscle (humero-antibrachialis) during limb regeneration while independently stimulating its own and an extensor nerve to the adjacent anconeus muscle. In each experiment, about 20 flexor muscle fibers were recorded consecutively from the edge that was adjacent to the antagonistic extensor muscle. At mid-digit stages of limb regeneration 33 muscles (589 fibers) were sampled and 47 $\pm$ 5% of the fibers were found to have received inappropriate synaptic inputs from the extensor nerve. More than 95% of the fibers sampled responded to one or both nerves. Of the few fibers showing no response to nerve stimulation, it was not resolved whether they had not received innervation yet or whether their axons were damaged during dissection. Of the 47% of the fibers that had received foreign innervation, 71% of them were innervated by both flexor and extensor nerves. This figure represents an underestimate of the proportion of dually innervated fibers because of inherent difficulties in recording intracellularly from these muscle fibers while the correct nerve is stimulated. Muscle contraction in response to nerve stimulation often dislocated the electrode before an input could be ascertained. Curare was not used in these experiments to block muscle contraction for it might also obliterate the relatively smaller foreign responses. The above percentage of inappropriate innervation is high due to the method of sampling near the edge of the muscle which borders the neighboring antagonist and does not represent the actual proportion of inappropriate connections made within a given muscle. The overall percentage of mistakes made within a given muscle was much less and in some muscles, no mistakes in nerve connections could be found. Nevertheless at late digit stages of regeneration, roughly 2 months after mid-digit stages

the probability of finding foreign synapses on flexor muscle fibers decreased. In 26 muscles (534 fibers) sampled by the same method, only 18 $\pm$ 4% of flexor fibers were found to receive inappropriate synaptic input from the extensor nerve. One disadvantage with this system is that development of a full grown limb takes about a year. At the late digit stages when the terminal experiments were performed, neither the limb nor the muscle fiber has reached its adult size yet.

I have no evidence at present to rule out the possibility that the decrease in percentage of fibers with foreign innervation is simply the result of an increase in the total number of muscle fibers with no change in the absolute number of foreign synapses. Such an issue might best be resolved by counting the number of muscle fibers with selective labelling of those that have received foreign innervation. Tetanic stimulation of motor axons has been found to deplete muscle glycogen (cf. Kugelberg, 1976). Perhaps tetanic stimulation of the foreign nerve would reveal the fibers that had received foreign inputs, as indicated by their depleted glycogen content.

Because of the variability encountered in these results, it was important to make sure that elimination of inappropriate synapses indeed did occur in these regenerating fibers. In some muscles the flexor nerve was sectioned at the mid-digit stages of limb regeneration. Work still in progress shows that within 1 month after lesion of the flexor nerve 72% of 751 flexor fibers sampled from 37 muscles were innervated by the extensor nerve, presumably by collateral sprouting. These muscles were reinnervated by the flexor nerve and when examined at the late digit stages of limb regeneration, the percentage of fibers innervated by the extensor n. was much decreased. 15 muscles (304 fibers) were sampled 2 - 6 months after correct denervation, 39% of the fibers were found to have residual foreign transmission.

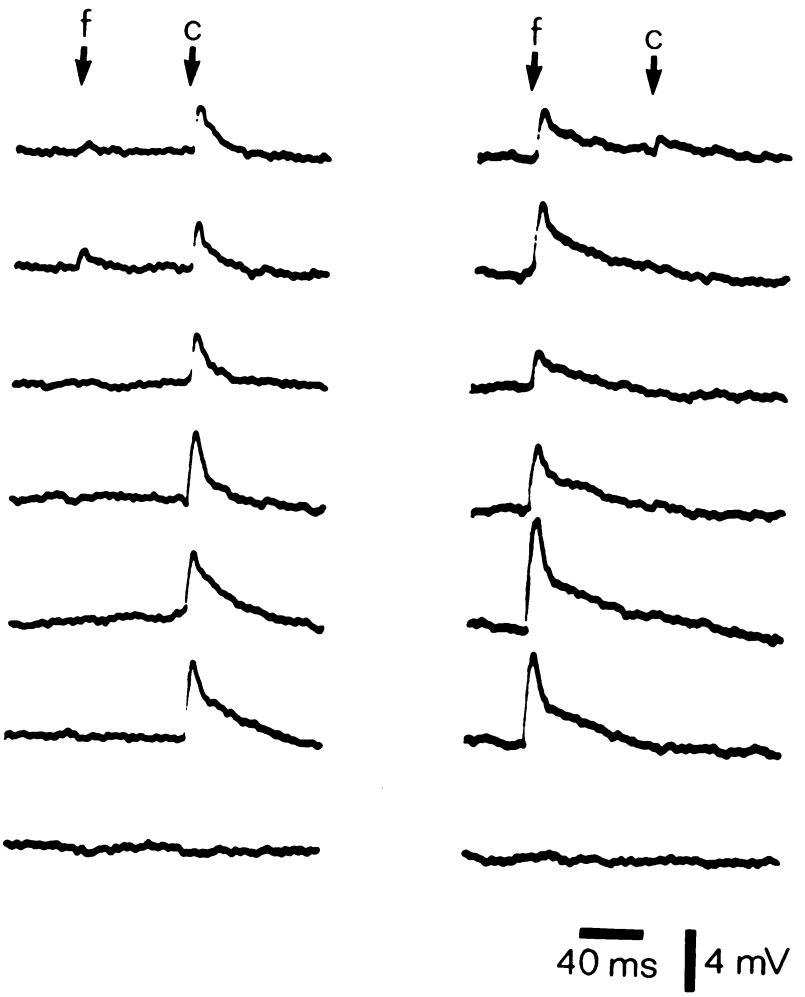
Although the relative amplitudes of correct and foreign input in dually innervated fibers might be important in elucidating a mechanism of foreign synapse suppression, no quantitative information

has yet been obtained due to the technical difficulties in recording from regenerating muscle fibers. Suffice it to say that not all inappropriate connections were subthreshold and that inappropriate connections were found on some fibers in which the correct was eliciting action potentials.

Relative Locations of Correct and Foreign Synapses. From a mechanistic point of view, it is of interest to know whether elimination of foreign synapses requires direct contact between correct and foreign terminals, or whether the competing synapses are physically separate from one another, In the present study physiological criteria were used to distinguish the relative locations of correct and foreign terminals. An end-plate in a fiber innervated dually by correct and foreign axons was localized as described in methods. A Ca^{++} solution was applied focally to that site in a Ca^{++} free medium while simultaneously stimulating the correct and the foreign nerves. The difference in latencies to onsets of correct and foreign responses following application of a pulse of calcium indicates the correct and foreign terminals are physically separate from one another (Fig. 6). At all 35 terminals mapped in this manner, the initial responses after Ca^{++} application were always obtained from only one or the other nerve. These results suggest that correct and foreign axons do not require direct contact for foreign suppression to occur.

Figure 6

Correct and foreign evoked end-plate potentials in response to focal Ca^{++} application at a synapse obtained from a fiber in a regenerating salamander limb. The sweeps are successive responses going from bottom to top. First the foreign and then 50 msec later the correct nerve was stimulated as indicated by the arrows in Ca^{++} free Ringer. This pattern was repeated every 2 seconds. At the start of the record, a brief pulse of Ca^{++} was applied focally at the synapse. The record on the left shows that focal Ca^{++} application at that site resulted in correct responses within 2 seconds, and only a small foreign response after 10 seconds, presumably due to diffusion of Ca^{++} to the distant foreign terminal. On the other hand, when in this case, the Ca^{++} pipette was moved to the other end of the microscopic field, 400 μm away, focal Ca^{++} application resulted in a foreign response within 2 seconds and a correct response after 12 seconds. These records demonstrate that correct and foreign terminals are physically separate from one another.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c
f
c
40 ms
4 mV

DISCUSSION

These results indicate that in adult newt muscle foreign synapses are suppressed as a consequence of correct reinnervation. This suppression involves a decline in the quantal content of transmitter released, with no change in the size of the quantal packets or in the postsynaptic transmitter sensitivity. In most instances this reduction of transmission went to completion and the foreign terminals were physically eliminated. Thirty-five percent of the fibers examined at long times (6 - 12 months) after correct reinnervation did retain some foreign synaptic function. However, transmission on these fibers too was reduced in that the evoked foreign responses were only a few millivolts in amplitude, in contrast to the suprathreshold foreign responses seen prior to correct reinnervation. The present results do not resolve whether the observed decline in quantal content occurs prior to terminal elimination or whether that decline occurs as the terminals are lost.

Another situation where suppression of foreign transmission occured was found in the regenerating salamander limb. During early stages of limb regeneration, inappropriate neuromuscular synapses were sometimes formed spontaneously, but were eliminated later in development. Thus synapse suppression is used during regeneration in adults to enhance the precision of nerve-muscle connections in salamander.

One would like to know the nature of the signal which initiates the loss of foreign transmission. One possibility is that there is direct contact between foreign and correct terminals which somehow triggers foreign nerve withdrawl. However my experiments on errors of innervation made spontaneously during salamander limb regeneration indicate that correct and incorrect synapses occur at separate sites on single muscle fibers, even in situation in which the incorrect synapses appeared to be suppressed. This suggests the alternative possibility, that the signal for withdrawl of

incorrect synapses is mediated by the target muscle fiber. Such a signal might involve the pattern of electrical activity of the muscle communicated via a feedback loop through muscle stretch receptors to motoneurones. An incorrect pattern of sensory feedback to a motoneurone may indicate maladaptive connections and somehow initiate withdrawl of their terminals. This scheme would require that foreign synaptic transmission be subthreshold so that it does not cause contraction and affect the normal loop of sensory-motor connections. The difficulty with this model is that a motoneurone has to distinguish its "correctness" from activities in one or a few 1A afferents, which represent only a small proportion of its many normal inputs. In addition, there is no evidence that a synapse may be confirmed or rejected simply by its pattern of stimulation. Nevertheless some indication for or against this possibility might be obtained by determining if sensory feedback plays a role in synapse suppression.

Another scheme by which synapses could be confirmed or rejected would be mediated by the presence or absence of a chemical substance secreted by the target cell. Such a chemical might be secreted by denervated muscles and promote the retention of foreign innervation. Upon correct reinnervation, the muscle might stop secreting this chemical and this might lead to withdrawl of the foreign synapses.

A third possible mechanism by which foreign synapses could be eliminated might be mediated by a chemical released from correct nerve terminals. The presence of this chemical might render the muscle membrane refractory to further innervation and induce it to reject incorrect synapses already formed. This notion is supported by the following lines of evidence: 1) A muscle fiber will not accept foreign innervation in the presence of correct innervation. 2) Muscle fibers become susceptable to foreign innervation once their correct inputs are eliminated. 3) Foreign innervation does not prevent correct axons from reinnervating their normal targets. In a recent study, Grinnel et al. (1977) presented evidence that innervation by one foreign nerve could not prevent another foreign

nerve from innervating the same muscle fibers in the frog. 4) Given an equal chance to compete, correct innervation is always favored over the incorrect. The existing evidence indicates that there are intrinsic differences between correct and foreign axons to a given target. It is quite possible that upon correct matching between a motoneurone and its target muscle fiber, muscle membrane function is changed and becomes refractory to innervation by inappropriate axons. The problem with this scheme is that one either has to propose a different chemical released by each type of motoneurone or that some sort of recognition between motoneurone and muscle fiber occur before a single chemical substance is released by the correct nerve terminals which then renders the muscle membrane refractory to all other innervation.

Whether sensory feedback and the pattern of electrical activity of the muscle are involved with synapse suppression can certainly be tested physiologically. More worthwhile experiments are required before further speculation about the mechanism is warranted.

The next level of inquiry regarding this process of synapse suppression concerns the actual cause for the loss of foreign transmission. Again there is little relevant information available at the present time. It has been proposed on the basis of indirect evidence (Harris, Wigston & Ziskind, 1977) that suppression of release involves a disruption of the entry of calcium into the axon terminal, which is required for normal secretion. A second possibility is that the decline in release is the result of terminal withdrawl, such that reduction in quantal content is a direct reflection of reduction in the area of contact between nerve and muscle. This would thus be correlated with a reduction in the number of release sites while the number of quanta released per site would remain fixed. Unfortunately the HRP labelling technique did not allow us to unequivocally distinguish correct from foreign terminals at the time when correct reinnervation was in progress (see results) and therefore cannot be used in the investigation of this problem. Hopefully further experiments will elucidate the mechanism underlying the suppression of foreign transmission.

The conclusion that suppressed synapses are eliminated from the muscle is in striking contrast to the claim of Cass et al. (1973) that suppressed synapses are morphologically normal. Two lines of evidence led Mark and his collaborators to believe that there were silent synapses in cross-innervated muscles: 1) the absence of degenerating synapses during correct reinnervation, and 2) the presence of both normal and degenerating synapses several days after a second correct nerve cut. These observations may be explained without invoking the presence of "silent" synapses. First, upon correct reinnervation, foreign terminals may retract without leaving recognizable degeneration products. Such a mechanism has been indicated in the elimination of polyneuronal innervation of neonatal rat skeletal muscle, in that no morphological signs of synapse degeneration were seen at a time when the number of transmitting synapses was being reduced (Korneliussen & Jansen, 1976). Second, the morphologically normal terminals in cross-innervated goldfish fibers observed several days after the correct nerve was cut (Mark et al., 1972) may have been those of functional foreign synapses: the behavioral test used in that study was not sufficiently sensitive to reveal subthreshold synaptic transmission (see results). More recently, Scott (1975) has questioned whether suppression of foreign transmission occurs at all in goldfish extraocular muscle. She believes that Mark's observations are explained by the regeneration of the excised muscle.

Only 36% of the muscle fibers examined had functional foreign innervation soon after a second correct nerve resection, 6 - 8 months after the first. Thus, a second correct nerve lesion produced no significant increase over the 35% residual foreign transmission seen without a second correct denervation, which was quite distinct from the complete foreign innervation seen after the first correct resection. This indicates that suppressed nerve terminals are not physically present in the muscle and able to take over fast. The fact that the residual function foreign terminals do not sprout to innervate all of the muscle fibers following the second degeneration of correct terminals is somewhat surprising. This may result because

the remaining foreign axons are extended beyond their normal scope of innervation and, as a consequence are not liable to further extension. Elimination of their correct inputs by excising their normal target muscle thus recreating the same situation as after the initial operation may reveal whether overextension is the explanation for the failure of residual foreign terminals to take over the rest of the muscle fibers.

Such phenomenon does not, however, seem to be the primary cause of foreign suppression; suppression was well correlated with the time of correct reinnervation, varying according to whether the correct nerve was crushed or resected, regardless of the regeneration and subsequent reinnervation of the normal target of the foreign nerve (m. humero-antibrachialis). Furthermore, in two muscles the correct nerve failed to reinnervate and in those the extent and efficacy of foreign transmission remained high even though the normal target of the foreign nerve was well regenerated and innervated.

Unlike the situation described here, adult mammalian muscle appears unable to reject foreign innervation once it has been established (Bernstein & Guth, 1961; Frank et al., 1974; Brown et al., 1976). Urodele amphibia retain in adulthood several embryonic characteristics and in this category perhaps belongs the ability to distinguish foreign from correct synaptic inputs and to suppress the foreign. Consistent with this idea is recent evidence that during newt limb regeneration inappropriate neuromuscular synapses sometimes form but are subsequently removed in favor of the correct ones. Some selectivity in retention of synapses also occurs in adult anuran amphibia. During reinnervation of frog and toad muscles, slow muscle fibers initially receive input from fast motor axons (Schmidt & Stefani, 1976), but this is subsequently replaced by the appropriate slow innervation (Hoh, 1971; Schmidt & Stefani, 1976). Also upon innervation of ectopically placed frog muscles by two incorrect nerves, there occurs a sorting out of fields such that each nerve synapses with some of the fibers yet with little overlap in the territory of the other nerve (Grinnell et al., 1976).

The ability of a target cell to distinguish between a variety of presynaptic inputs and eliminate some would be of obvious utility in development and molding of the central nervous system. The lack of suppression of inappropriate synapses experimentally induced on cells of adult mammals probably results from a loss during maturation of flexibility present in the developing nervous system; such a loss of plasticity with age has been clearly shown in the mammalian visual system (Hubel & Wiesel, 1970). Further examination of normal embryonic development may reveal whether synapse suppression as described here is indeed put to use for enhancing the precision of excitable cell interaction.

REFERENCES

Arora, H. L. and Sperry, R. W. (1957). Myotypic respecification of regenerated nerve-fibers in Cichlid fishes. J. Embryol. Exp. Morph. 5, 256-263.

Bagust, J., Lewis, D. M. and Westerman, R. A. (1973). Polyneuronal innervation of kitten skeletal muscle. J. Physiol. 229, 241-255.

Bernstein, J. J. and Guth, L. (1961). Nonselectivity in establishment of neuromuscular connections following nerve regeneration in the rat. Exp. Neurol. 4, 262-275.

Brown, M. C., Jansen, J. K. S. and Van Essen, D. (1976). Polyneuronal innervation of skeletal muscle in newborn rats and its elimination during maturation. J. Physiol. 261, 387-422.

Cass, D. T., Sutton, T. J. and Mark, R. F. (1973). Competition between nerves for functional connections with axoltl muscles. Nature, Lond. 243, 201-203.

Close, R. (1964). Dynamic properties of fast and slow skeletal muscles of the rat during development. J. Physiol. 173, 74-95.

del Castillo, J. & Katz, J. (1954). Quantal components of the end-plate potential. J. Physiol. 124, 560-573.

Dennis, M. J. (1975). Physiological properties of junctions between nerve and muscle developing during salamander limb regeneration. J. Physiol. 244, 683-702.

Dennis, M. J. & Miledi, R. (1974). Non-transmitting neuromuscular junctions during an early stage of end-plate reinnervation. J. Physiol. 239, 553-570.

Elul, R., Miledi, R. and Stefani, E. (1970). Neural control of contracture in slow muscle fibres of the frog. Acta Physiol. Latinoamer. 20, 194-226.

Fangboner, R. F. & Vanable, J. W. (1974). Formation and regression of inappropriate nerve sprouts during trochlear nerve regeneration in Xenopus laevis. J. Comp. Neur. 157, 391-406.

Fatt, P. and Katz, B. (1951). An analysis of the end-plate potential recorded with an intracellular electrode. J. Physiol. 115, 320-370.

Frank, E. and Jansen, J. K. S. (1976). Interaction between foreign and original nerves innervating gill muscles in fish. J. Neurophysiol. 39, 84-90.

Frank, E., Jansen, J. K. S., Lømo, T. & Westgaard, R. H. (1975). The interaction between foreign and original motor nerves innervating the soleus muscle of rats. J. Physiol. 247, 725-743.

Gaze, R. M. (1970). The Formation of Nerve Connections. Academic Press, London.

Graham, R. C. & Karnovsky, M. J. (1966). The early stages of absorption of injected horseradish peroxidase in the proximal tubule of the mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14, 291-302.

Grimm, L. M. (1971). An evaluation of myotypic respecification in axolotls. J. Exp. Zool. 178, 419-496.

Grinnell, A. D., Rheuben, M. B. & Letinsky, M. S. (1977). Mutual repression of synaptic efficacy by pairs of foreign nerves innervating frog skeletal muscle. Nature, Lond. 265, 368-370.

Harris, A. J. (1974). Inductive functions of the nervous system. A. Rev. Physiol. 36, 251-305.

Harris, A. J., Wigston, D. and Ziskind, L. (1977). Spontaneous release of transmitter from "repressed" nerve terminals in axoltl muscle. Nature, Lond. (in press).

Heuser, J. E. & Reese, T. S. (1973). Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J. Cell Biol. 57, 315-344.

Hoh, J. F. Y. (1971). Selective reinnervation of fast-twitch and slow-graded muscle fibers in the toad. Exp. Neurol. 30, 263-276.

Hoh, J. F. Y. (1975). Selective and non-selective reinnervation of fast-twitch and slow-twitch rat skeletal muscle. J. Physiol. 251, 791-801.

Hubel, D. H. & Wiesel, T. N. (1970). The period of susceptibility to the physiological effects of eye closure in kittens. J. Physiol. 206, 419-436.

Iten, L. E. and Bryant, S. V. (1973). Forelimb regeneration from different levels of amputation in the newt, Notopthalmus

viridescens: length, rate and stages. Wilhelm Roux Arch. Entw Mech. 173, 263-282.

Jacobson, M. (1970). Developmental Neurobiology. Holt, Rinehart & Winston, New York.

Korneliussen, H. & Jansen, J. K. S. (1976). Morphological aspects of the elimination of polyneuronal innervation of skeletal muscle fibres in newborn rats. J. Neurocytol. 5, 591-604.

Kuffler, S. W. and Vaughan Williams, E. M. (1953). Properties of the "slow" skeletal muscle fibres of the frog. J. Physiol. 121, 318-340.

Kugelberg, Eric (1976). Adaptive transformation of rat soleus motor units during growth. J. Neurol. Sci. 27, 269-289.

Landmesser, L. and Morris, D. G. (1975). The development of functional innervation in the hind limb of the chick embryo. J. Physiol. 249, 301-326.

Lehouelleur, J. & Chatelain, A. (1974). Analysis of electrical responses of newt skeletal muscle fibres in response to direct and indirect stimulation. J. Physiol., Paris, 68, 615-632.

Lentz, T. L. (1969). Development of the neuromuscular junction. I. Cytological and cytochemical studies on the neuromuscular junction of differentiating muscle in the regenerating limb of the newt Triturus. J. Cell Biol. 42, 431-443.

Mark, R. F. (1965). Fin movement after regeneration of neuromuscular connections: an investigation of myotypic specificity. Exp. Neurol. 12, 292-302.

Mark, R. F. (1974). Memmory and Nerve Connections. Clarendon Press, Oxford.

Mark, R. F. and Marotte, L. R. (1972). The mechanism of selective reinnervation of fish eye muscles. III. Functional, electrophysiological and anatomical analysis of recovery from section of IIIrd and IVth nerves. Brain Res. 46, 131-148.

Mark, R. F., Marotte, L. R. and Mart, P. E. (1972). The mechanism of selective reinnervation of fish eye muscles. IV. Identification of repressed synapses. Brain Res. 46, 149-157.

Marotte, L. R. and Mark, R. F. (1970a). The mechanism of selective

reinnervation of fish eye muscle. I. Evidence from muscle function during recovery. Brain Res. 19, 41-51.

Marotte, L. R. and Mark, R. F. (1970b). The mechanism of selective reinnervation of fish eye muscle. II. Evidence from electron-microscopy of nerve endings. Brain Res. 19, 53-62.

Miledi, R. and Orkand, Paula. (1966). Effect of a 'fast' nerve on 'slow' muscle fibres in the frog. Nature, Lond., 209, 717-718.

Purves, D. (1976). Long-term regulation in the vertebrate peripheral nervous system; in, Review of Physiology, Vol. 10, Neurophysiology 2, (Ed. R. Porter), University Park Press, Baltimore, pp. 125-178.

Redfern, P. A. (1970). Neuromuscular transmission in new-born rats. J. Physiol. 209, 701-709.

Rosenthal, J. L. and Taraskevich, P. S. (1977). Reduction of multiaxonal innervation at the neuromuscular junction of the rat during development. J. Physiol. (in press).

Schmidt, H. and Stefani, E. (1976). Re-innervation of twitch and slow muscle fibres of the frog after crushing the motor nerves. J. Physiol. 258, 99-123.

Scott, S. A. (1975). Persistence of foreign innervation on reinnervated goldfish extraocular muscles. Science, N. Y., 189, 644-646.

Sperry, R. W. (1945). The problems of central nervous reorganization after nerve regeneration and muscle transposition. Qu. Rev. Biol. 26, 314-363.

Sperry, R. W. (1947). Nature of functional recovery following regeneration of the oculomotor nerve in amphibians. Anat. Rec. 97, 293-316.

Sperry, R. W. (1950). Myotypic specificity in teleost motoneurones. J. Comp. Neurol. 93, 277-288.

Sperry, R. W. (1951). Mechanisms of neural maturation; in, Handbook of Experimental Psychology. (Ed. S. S. Stevens). Wiley, New York, pp. 236-280.

Sperry, R. W. and Arora, H. L. (1965). Selectivity in regeneration of the oculomotor nerve in the cichlid fish, Astronotus ocellatus.

J. Embryol. exp. Morph. 14, 307-317.

Sperry, R. W. and Deupree, N. (1956). Functional recovery following alterations in nerve-muscle connections of fishes. J. Comp. Neurol. 106, 143-161.

Tonge, D. A. (1974). Synaptic function in experimental innervated muscle in the mouse. J. Physiol. 239, 968.

Weiss, P. (1922). Die Funktion transplantierten Amphibienextremitaten. Ang. Akad. Wiss. Wien, 59, 199-201.

Weiss, P. (1926). The relation between central and peripheral coordination. J. Comp. Neurol. 40, 241-251.

Weiss, P. (1936). Selectivity controlling the central-peripheral relations in the nervous system. Biol. Rev. 11, 494-531.

Weiss, P. (1937a). Further experimental investigations on the phenomenon of homologous response in transplanted amphibian limbs. I. Functional observations. J. Comp. Neurol. 66, 181-209.

Weiss, P. (1937b). Further experimental investigations on the phenomenon of homologous response in transplanted amphibian limbs. II. Nerve regeneration and the innervation of transplanted limbs. J. Comp. Neurol. 66, 481-535.

Weiss, P. (1937c). Further experimental investigations on the phenomenon of homologous response in transplanted amphibian limbs. III. Homologous response in the absence of sensory innervations. J. Comp. Neurol. 66, 537-548.

Weiss, P. (1937d). Further experimental investigations on the phenomenon of homologous response in transplanted amphibian limbs. IV. Reverse locomotion after interchange of right and left limbs. J. Comp. Neurol. 67, 269-315.

Weiss, P. (1941). Self-differentiation of the basic patterns of coordination. Comp. Psychol. Monog. 17, 1-96.

Weiss, P. and Hoag, Ann. (1946). Competitive reinnervation of rat muscles by their own and foreign nerves. J. Neurophysiol. 9, 413-418.

Wiersma, C. A. G. (1931). An experiment on the "resonance theory" of muscular activity. Arch. Neerl. Physiol. 16, 337-345.