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NON-TRANSMITTING NEUROMUSCULAR JUNCTIONS DURING AN EARLY STAGE OF END-PLATE REINNERVATION

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SUMMARY

1. Electrophysiological studies were made on regenerating motor end-plates in frog cutaneous pectoris muscle after crushing the motor nerve.

2. The pattern of degenerative and regenerative changes is similar to that already described for the frog sartorius, although it occurs more quickly in the cutaneous pectoris. Resumption of synaptic transmission first occurs after 9 days, in muscle fibres closest to the nerve crush. During the subsequent 12 days synaptic contact is progressively re-established across the muscle.

3. During the period of regeneration muscle fibres can be found which have been reinnervated, but which do not produce an end-plate potential in response to stimulation of the nerve trunk. This is referred to as the 'non-transmitting' stage of regeneration.

4. Increases in extracellular potassium, addition of lanthanum to the bath and focal extracellular stimulation all indicate that the axon terminals at non-transmitting junctions do have a releasable supply of neurotransmitter. Focal stimulation occasionally sets up active responses in these nerve terminals.

5. It is concluded that this 'non-transmitting' stage results from failure of action potential propagation in the regenerating neurone proximal to the end-plate region.

INTRODUCTION

Study of the regeneration of neuromuscular contacts is of interest not only because it leads toward an understanding of the changes which occur during reformation of synapses, but also because it may shed some light on the more general problem of the development of synaptic connexions.

In a previous study of regeneration of neuromuscular junctions in the

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frog it was found that there is a period during which an axon has already established synaptic contact, but does not transmit excitation to the muscle fibre (Miledi, 1960). In this paper we consider in more detail the characteristics of transmitter release by nerve terminals which are at this 'non-transmitting' stage of regeneration, and compare them with those of normal neuromuscular junctions. In a subsequent paper we shall examine a later stage of regeneration in which transmission has been re-established, but differs from that of the normal adult junction in that the spontaneous release of transmitter seems to occur in abnormally small packets (Dennis & Miledi, 1974*b*).

METHODS

Most experiments were performed on the cutaneous pectoris muscle of the frog (*R. temporaria*). The cutaneous pectoris is a thin muscle (cf. Dennis & Miledi, 1974*a*) well suited for viewing the motor end-plates with Nomarski interference-contrast optics. Some experiments were also carried out on the sartorius muscle of the frog, but there interference optics are only useful at the edges, where the muscle is one or two fibres thick. Denervation was performed under ether anaesthesia by crushing the motor nerves approximately 1 mm from their entry to the muscles; the closest end-plates were located 2–3 mm from the crush in both muscles. All animals were subsequently kept at room temperature (20–23° C).

In addition, a few experiments were carried out using the rat diaphragm. The denervation was performed under ether anaesthesia by crushing the left phrenic nerve in the thorax, near its entry to the muscle.

The methods used have been previously described (cf. Dennis & Miledi, 1974*a*). The composition of the bathing fluid was altered according to the specific requirements of each experiment. The standard solution had the following composition (m-mole): Na^+, 132; K^+, 2; Ca^{2+}, 1·8; Cl^-, 130·72; $H_2PO_4^-$, 1·12; HPO_4^{2-}, 2·88; glucose, 10. For the rat diaphragm, we used a modified Krebs solution, equilibrated with 95% O_2/5% CO_2, of the following composition: Na^+, 150; K^+, 4; Ca^{2+}, 2; Mg^{2+}, 1; Cl^-, 147; $H_2PO_4^-$, 1; HCO_3^-, 12; glucose, 10. The anticholinesterase agent neostigmine methylsulphate was usually added in a concentration of 10^{-6} g/ml. When lanthanum was used, the muscle was first equilibrated in buffer-free Ringer, the lanthanum was then applied in the unbuffered solution to avoid precipitation (cf. Heuser & Miledi, 1971).

In a few experiments which required verification that an end-plate had been reinnervated, the muscle was fixed and stained with ZnI_2–OsO_4 (Akert & Sandri, 1968) at the end of the experiment. This was a reliable method of confirming that the nerve projections seen on a living fibre were accurate.

RESULTS

Morphology

Nomarski optics were used in this investigation because they improve the resolution of the unmyelinated nerve terminal at the motor end-plate (McMahan, Spitzer & Peper, 1972) as well as on frog autonomic ganglion cells (McMahan & Kuffler, 1971). In earlier studies of normal neuro-

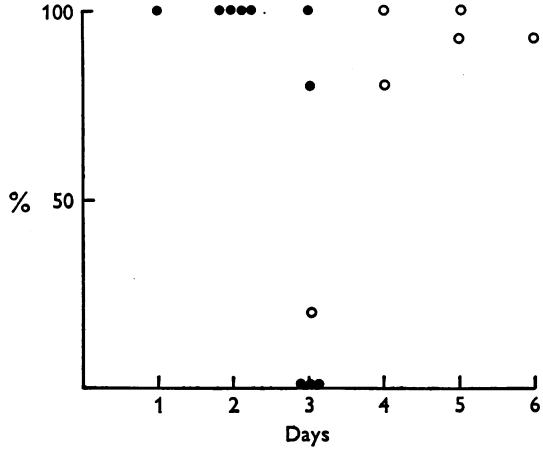
muscular junctions using conventional microscopy (Katz & Miledi, 1965) it was not possible to resolve routinely the projections of the nerve terminal beyond the last myelin segment, although some unmyelinated branches could frequently be seen. Even with Normarski optics one cannot visualize the terminals of every end-plate; however, at surface junctions in regions where the muscle is only one or a few fibres thick it is possible to see most of the unmyelinated nerve terminal branches (Pl. 1*A*).

When looking at the end-plate region of living fibres, as in Pl. 1*A*, the question arises whether one is viewing not so much the axon terminal itself as the trough in the muscle in which it lies, or the projection of the Schwann cell covering the axon. If this were so, then the junction might look the same after degeneration of the nerve terminal, making it difficult to judge by microscopic inspection when the regenerating axon had returned. Fortunately, this is not the case. Upon degeneration of the motor terminal subsequent to cutting, or crushing, the nerve, the characteristic appearance of the terminals disappears (Pl. 1 *B*), even though the synaptic groove and Schwann cells remain after the axon degenerates (Birks, Katz & Miledi, 1960). In that condition the end-plate region can still be recognized by the remnants of the degenerating myelin and by the Schwann cell nuclei, but the tube-like aspect of the unmyelinated nerve is gone. Upon reinnervation of an end-plate, as judged by physiological or histological criteria, it is again possible to see the projections of the nerve in the end-plate region of the living muscle fibres (Pl. 1*C*).

Time course of degeneration

When the nerve to the frog sartorius muscle is cut, action potential propagation and transmitter release are maintained in the distal stump for 4 or 5 days, after which transmission fails. At this time the spontaneous miniature end-plate potentials (min. e.p.p.s) also cease. The length of time that transmission is maintained after nerve severance depends on both temperature and the length of nerve left attached to the muscle (Birks *et al.* 1960; Miledi & Slater, 1970). The ensuing 'silent period' lasts for several days and is followed by a gradual resumption of local spontaneous activity at the denervated end-plates due to quantal release of acetylcholine from the Schwann cells (Birks *et al.* 1960). Degeneration of the nerve to the cutaneous pectoris follows a similar pattern, but the changes seem to occur more rapidly; transmission and spontaneous release of acetylcholine fail between the second and fourth day (Text-fig. 1). This difference in the onset of neuromuscular failure in sartorius and cutaneous pectoris muscles is probably due to a difference in the distance of the end-plates from the point at which the nerve is severed. However, since large diameter motor axons degenerate more quickly than small

(Katz & Miledi, 1959; Elul, Miledi & Stefani, 1968), and since there seem to be seasonal variations in the rate of end-plate degeneration (Harris & Miledi, 1972), it is possible that these factors are also involved.



Text-fig. 1. Occurrence of spontaneous miniature end-plate potentials in cutaneous pectoris muscles after crushing the nerve. Each point represents the results from one muscle, derived by recording at the end-plates of at least twenty fibres for 1–2 min each. Abscissa, time in days after crushing the nerve. Ordinate, percentage of end-plates at which spontaneous potentials were observed. Filled circles, end-plates where the spontaneous potentials had a normal frequency range and amplitude distribution. Open circles, end-plates where spontaneous activity had the low frequency and skew amplitude distribution typical of denervated fibres.

As in the sartorius and other frog muscles, the failure of transmission in the cutaneous pectoris is followed by a 'silent period', lasting one or two days, during which no spontaneous miniature potentials are detectable. Subsequently, 4–5 days after nerve severance but before any nerve regeneration had occurred, we began to detect the infrequent min. e.p.p.s which result from release of acetylcholine quanta from the Schwann cells (Birks *et al.* 1960; Miledi & Slater, 1968; Bevan, Miledi & Grampp, 1973; Dennis & Miledi, 1974*a*). In a few muscles there was so much variability in the state of individual end-plates, that some fibres had normal-looking min. e.p.p.s, while others were in the 'silent period', and still others had what seemed to be 'Schwann min. e.p.p.s', as judged from their low frequency and skew amplitude distribution.

Restoration of neuromuscular transmission

The region where the nerve enters the muscle, the lateral edge, is also the thickest part. The first fibres to be functionally reinnervated occurred in this region and were often deep. In the present set of experiments, the

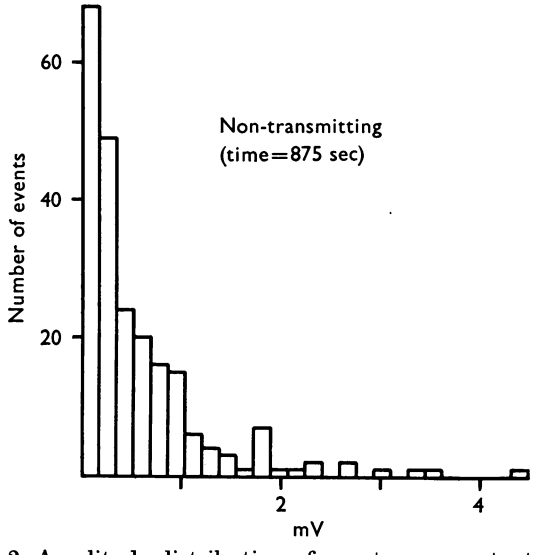
earliest time at which stimulation of the nerve trunk elicited a twitch in any of the muscle fibres was 9 days after nerve crush in the cutaneous pectoris and 11 days in the sartorius muscle. However, we know that there is a period of time after reinnervation of a fibre when stimulation of the nerve trunk does not yet elicit an end-plate potential (Miledi, 1960; see also below), so the earliest contact between nerve and muscle must have been re-established sooner, at most 6–8 days after crushing the nerve. There is considerable variation from muscle to muscle in the time of resumption of synaptic transmission. In the cutaneous pectoris, the first fibre twitch in response to nerve stimulation generally occurred 9–12 days after the crush. Presumably, this variation is due at least in part to factors such as length of nerve segment distal to the crush, width of the crushed region, rate of healing of the incision in the skin and health of the frog.

Reinnervation of the muscle fibres progresses from the region of the nerve entry across the muscle towards the medial edge. However, within this general pattern there is considerable irregularity, such that often fibres which have been reinnervated are found further from the nerve entry than others which give no indication that the nerve has re-established synaptic contact. Neuromuscular transmission in the cutaneous pectoris is usually re-established in the most distant fibres between 17 and 22 days after crushing the nerve.

Initial signs of reinnervation

During neuronal regeneration the axons grow into the old motor end-plates, and re-establish synaptic contact. However, as with the frog sartorius (Miledi, 1960) the first indication of reinnervation of a muscle fibre is not the resumption of synaptic transmission in response to stimulation of the nerve trunk, but rather an increase in frequency of spontaneous min. e.p.p.s recorded at the old end-plate region. The rate of occurrence of Schwann min. e.p.p.s in a muscle which has been denervated 2–3 weeks is usually in the range of 0·25–10 min. During the early stages of reinnervation (9–20 days after crushing the nerve) end-plates are found which have a more normal frequency of min. e.p.p.s (approximately 10–60/min), but which do not show an end-plate potential (e.p.p.) when the nerve trunk is stimulated. This we refer to as the 'non-transmitting' stage of reinnervation. The fact that the frequency of min. e.p.p.s at such reinnervated junctions is usually lower than the average for normal terminals may simply reflect a smaller area of contact between axon terminal and muscle. The amplitudes of the spontaneous potentials recorded in such fibres are still unlike those seen at normal end-plates; the amplitude distribution is skewed towards the origin. Histograms of the amplitudes

of min. e.p.p.s recorded at reinnervated junctions which did not respond to nerve stimulation are illustrated in Text-figs. 2–4 and 6. In those cases where the end-plate of such a fibre was suitably located for microscopic inspection, one could see that the nerve terminal had in fact returned to the old end-plate.



Text-fig. 2. Amplitude distribution of spontaneous potentials recorded at a reinnervated end-plate which did not respond to nerve-trunk stimulation. Thirteen days after crushing the nerve. Resting potential, 88 mV. Recording time, 875 sec (min. e.p.p.s frequency, 15/min). Abscissa, amplitude of individual potentials; ordinate, number of potentials observed. Subsequent addition of lanthanum induced a large increase in frequency, confirming the presence of an axon terminal in the end-plate. Ringer contained neostigmine methylsulphate 10^{-6} g/ml. in this and all subsequent Text-figures, except Text-fig. 7.

The rate of occurrence of spontaneous min. e.p.p.s varies greatly in different frogs, such that there is some overlap in the frequency of Schwann min. e.p.p.s at denervated end-plates with that of spontaneous potentials at reinnervated junctions within the same muscle. Consequently the use of frequency alone is not a reliable indication of the presence or absence of a motor nerve terminal, except, of course, at the extremes encountered in some end-plates. The amplitude distributions of min. e.p.p.s recorded at normally innervated and at denervated end-plates are very different (Birks *et al.* 1960), but unfortunately at newly reinnervated junctions the spontaneous potentials often have a skewed amplitude distribution (Text-fig. 2) resembling that of Schwann min. e.p.p.s (cf. Dennis & Miledi, 1974*b*). For this reason the min. e.p.p. amplitude distribution alone was also not

sufficient to judge whether a junction had been reinnervated, except in occasional fibres which showed a 'mixed' histogram (cf. Miledi, 1960) containing both a skew and a normal portion, like that illustrated in Text-fig. 6. As mentioned above, it was sometimes possible to see the nerve terminal in a newly reinnervated fibre when the end-plate was on the surface. However, as none of these indices alone is reliable, we used a combination of the three to judge whether or not an old end-plate had received a new nerve terminal.

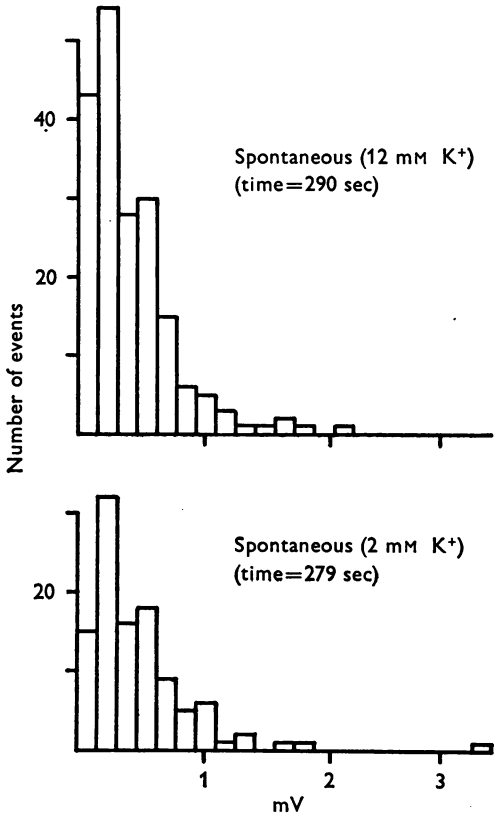
At slightly later stages of regeneration there is no ambiguity because stimulation of the nerve trunk does elicit a junctional potential at the reinnervated end-plate. Some characteristics of transmitter release during that phase will be considered in a subsequent paper (Dennis & Miledi, 1974*b*).

Spontaneous release of transmitter from non-transmitting reinnervated junctions

The frequency of min. e.p.p.s at normal end-plates is increased when either the potassium concentration or the tonicity of the bathing fluid is raised above normal, whereas, such changes have practically no effect, or may even reduce the frequency of Schwann min. e.p.p.s at denervated end-plates (Birks *et al.* 1960). This difference in the response to changes in the bathing medium can be used as further indication of the presence or absence of axon terminals at junctions which do not yet respond to stimulation of the nerve trunk. In fact, in an earlier study it was mentioned that hypertonic solutions increased the frequency of min. e.p.p.s in reinnervated junctions which were at the non-transmitting stage (Miledi, 1960).

Raising the concentration of potassium in the bathing fluid likewise caused an acceleration in the rate of min. e.p.p. discharge of non-transmitting junctions: a change from 2 to 12 mM $[K]_o$ caused the mean frequency to increase two- to tenfold, whereas the same change in normal control muscles caused increases of three- to tenfold. The actual increase in rate of transmitter release under these conditions may, in fact, be somewhat greater since the amplitudes of many min. e.p.p.s are close to the noise level and the muscle fibre depolarization in high $[K]_o$ could obscure some of the smallest min. e.p.p.s. This depolarization of the muscle fibre also makes it difficult to determine whether the increase in frequency is uniform for all min. e.p.p.s, or whether there is a relatively greater acceleration of the large ones, as is the case when lanthanum is added (see below). One fibre had a low resting potential upon penetration, and an increase in $[K]_o$ from 2 to 12 mM caused little further depolarization, making it possible to compare the distribution in amplitude of min. e.p.p.s recorded at the two potassium concentrations (Text-fig. 3). As can be

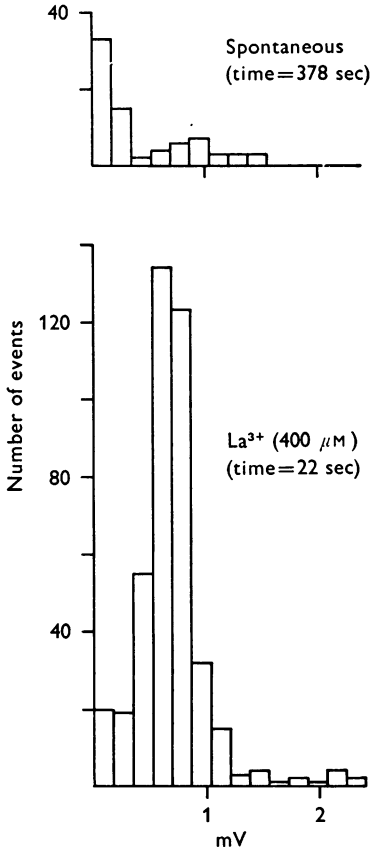
seen, there was no great change in the mean amplitude or distribution of the min. e.p.p.s with the doubling of frequency caused by the increased potassium.



Text-fig. 3. Effect of potassium concentration on the frequency and amplitude of spontaneous potentials recorded from a non-transmitting end-plate. Bottom, amplitude histogram in normal potassium (2 mM); recording time, 279 sec; resting potential, 55 mV. Top, amplitude histogram in 12 mM potassium; recording time, 290 sec; resting potential, 45 mV. Fourteen days after crushing the nerve.

Another agent which increases the rate of spontaneous release at normal terminals but not that from Schwann cells, is lanthanum. Micromolar concentrations in the bathing fluid cause a marked increase in the frequency of min. e.p.p.s at normal end-plates (Blioch, Glagoleva, Liberman & Nenashev, 1968; Heuser & Miledi, 1971; DeBassio, Schnitzler & Parsons, 1971), but have no effect on the frequency of min. e.p.p.s at denervated end-plates. When lanthanum was applied to reinnervated muscles in concentrations of 50–500 μM, the frequency of min. e.p.p.s at transmitting

junctions was increased by factors of 4·3–190 times (six experiments). This procedure produced comparable effects at non-transmitting junctions, as illustrated in Text-fig. 4, where 400 μM lanthanum caused an increase in min. e.p.p. frequency of approximately 100-fold. Note that there was a relatively greater acceleration of the larger potentials, although even the



Text-fig. 4. Effect of lanthanum on the frequency and amplitude of spontaneous potentials recorded from a non-transmitting end-plate. Top, buffer-free saline, time 378 sec; bottom, after addition of 400 μM lanthanum, time 22 sec. Resting potential, 90 mV.

smallest recorded potentials did increase in frequency. In a few fibres thought to be reinnervated, the addition of lanthanum caused no change in the min. ep.p.s frequency. In these fibres the initial criterion for reinnervation was solely the fact that the min. e.p.p. frequency was somewhat higher than average for denervated fibres, which is not always a reliable index. The lack of effect of lanthanum in those exceptional cases

could have resulted if the fibres had, in fact, not yet been reinnervated: alternatively, at the earliest stage of reinnervation La-treatment may not influence spontaneous transmitter release.

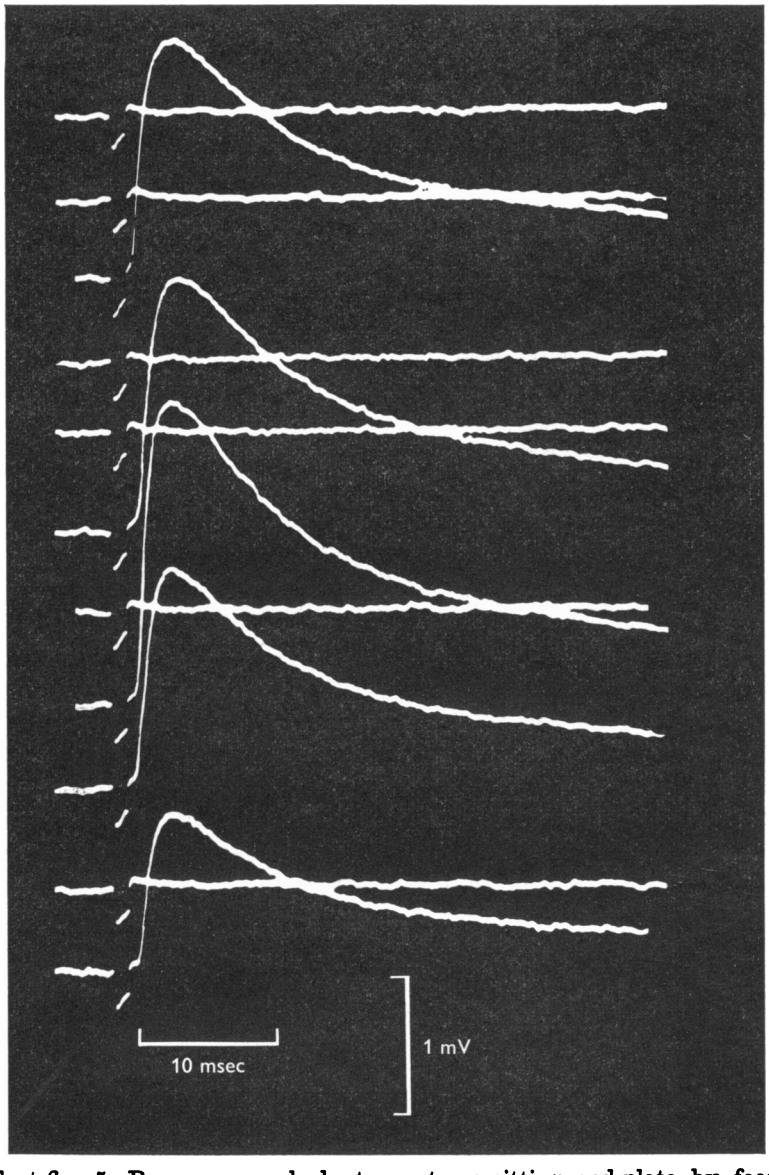
The finding that, in general, non-transmitting junctions do respond to addition of potassium or lanthanum indicates that the elevated min. e.p.p. frequencies are due to an increase in quanta of transmitter being released from the reinnervating axon terminals.

Focal electrical stimulation of regenerated nerve terminals

The lack of response to nerve stimulation at recently reinnervated end-plates has several possible explanations. One is that the nerve action potential fails to invade the nerve terminal, as was suggested previously (Miledi, 1960). Alternatively, the axon may be able to conduct impulses to the terminals as soon as it reinnervates the end-plate, but the coupling between membrane depolarization and transmitter release could initially be weak or absent, and develop only later.

The increase in min. e.p.p. frequency with raised external potassium suggests that some electro-secretory coupling has already developed in non-transmitting end-plates. Further proof was obtained by stimulating nerve terminals at non-transmitting junctions with a focal extracellular micropipette. A relatively blunt, 2–4 μm tip, electrode filled with sodium chloride, was placed on the nerve terminal of a non-transmitting junction and brief pulses of depolarizing current were applied while recording intracellularly from the muscle fibre with a second micropipette. If the failure of neuromuscular transmission were due to failure of impulse propagation then focal stimulation would be expected to elicit transmitter release, as at normal junctions (Katz & Miledi, 1967). Whereas, if the impulse invades but there is no electro-secretory coupling, then direct electrical stimulation of a nerve terminal would not elicit a response in the muscle fibre except, perhaps, under the extreme conditions of dielectric break-down of the membrane (del Castillo & Katz, 1954*b*).

To test these possibilities, experiments were made on exposed non-transmitting end-plates in which at least part of the newly grown axon was visible. Intracellular recording was used to assess the stage of re-innervation. End-plates were deemed to be in the non-transmitting stage if the frequency of min. e.p.p.s was higher than the frequency of Schwann min. e.p.p.s usually observed at denervated end-plates, and if the end-plates failed to respond to repeated stimulation of the nerve trunk. The nerve stimulation was carried out at normal calcium concentration to ensure that any end-plate response would be easily detected. One difficulty, however, was that the stimulation caused movement artifacts due to contraction of those muscle fibres where adequate transmission had already



Text-fig. 5. Responses evoked at non-transmitting end-plate by focal stimulation of a regenerated nerve terminal. Stimulus pulses, indicated by artifact, of constant duration and amplitude were repeated once every 2 sec. As can be seen, the responses were intermittent and fluctuated in amplitude. Fourteen days after crushing nerve.

been re-established. To reduce this complication muscles were used early during regeneration, 9–15 days after the operation, when only a few junctions were transmitting. At such a period no, or at most only a few, exposed, non-transmitting end-plates were found in any one muscle. In all, thirty-four end-plates from twenty-one muscles met our requirements.

Once an exposed, non-transmitting end-plate was identified, the tip of the stimulating pipette was placed against the observed axon terminal and negative pulses of 1–2 msec were applied with gradually increasing intensity until either a response was elicited or the current electrode penetrated the muscle fibre (cf. Katz & Miledi, 1967). By stimulating nerve terminals of non-transmitting junctions in this manner, it was possible to evoke the release of small amounts of transmitter (Text-fig. 5) from eighteen of the thirty-four fibres studied. The resultant potentials looked very much like the quantal responses recorded from the end-plate region of normal muscle fibres (del Castillo & Katz, 1954*a*); their amplitudes were in the range of one half to several millivolts, they fluctuated in steps which appeared to be quantal, and their time courses were similar to those of spontaneous min. e.p.p.s recorded at the same ending.

On a few occasions, during the placement of the stimulating pipette we set off a barrage of spontaneous min. e.p.p.s which continued at a high frequency for some minutes. It is probable that this resulted from mechanical damage to the nerve terminal by the electrode tip. Such observations provide further evidence that non-transmitting terminals are capable of releasing packages of transmitter.

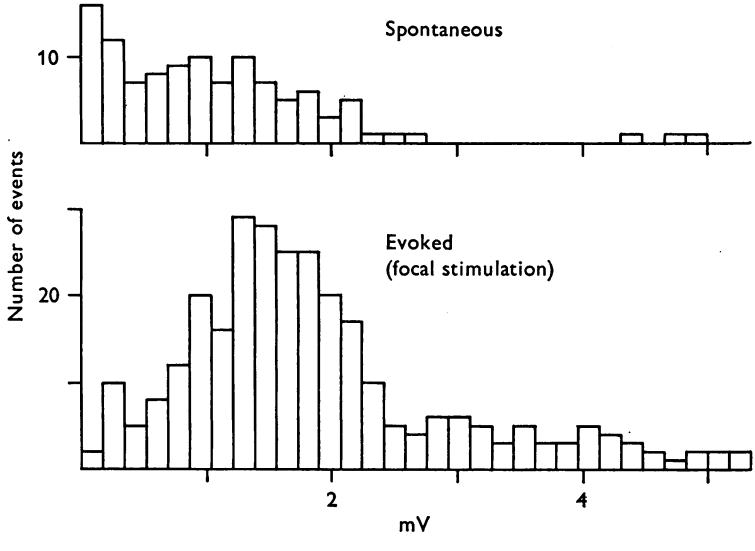
In sixteen out of eighteen non-transmitting end-plates stimulated focally the response was not maintained for more than twenty or thirty pulses. Presumably this liability resulted because the newly regenerated nerve terminal is smaller and more susceptible to damage than normal. At two end-plates, however, stimulation of the terminal was sustained and we obtained a sufficiently large sample of responses to plot a histogram of their amplitudes (Text-fig. 6). As can be seen, the amplitudes of these responses had a bell-shaped distribution like those elicited at normal junctions by either stimulating the nerve trunk in reduced calcium (del Castillo & Katz, 1954*a*) or by focally stimulating the terminal in the presence of tetrodotoxin (Katz & Miledi, 1967).

Although no systematic study was made of the synaptic delay of these end-plate potentials evoked by focal stimulation, it did not appear to be very much longer than normal. Also, there was no obvious evidence that the double and triple unit responses elicited in this manner had a longer delay.

As described in a preceding paper (Dennis & Miledi, 1974*a*), focal extracellular stimulation of Schwann cells at denervated end-plates also causes the release of

ACh. However, the responses to Schwann cell stimulation are characteristically different from those described here; the Schwann e.p.p. is always slower than that of the spontaneous min. e.p.p.s recorded at the same junction, whereas the e.p.p. evoked from the nerve terminal has a rising phase resembling that of the min. e.p.p.s. Besides rising more slowly, the Schwann release is of much longer duration – the depolarization sometimes lasts more than 500 msec. Furthermore, the axonal transmitter release fluctuates in steps which appear to be quantal, whereas the Schwann release seems to be continuously graded, or at least composed of smaller subunits.

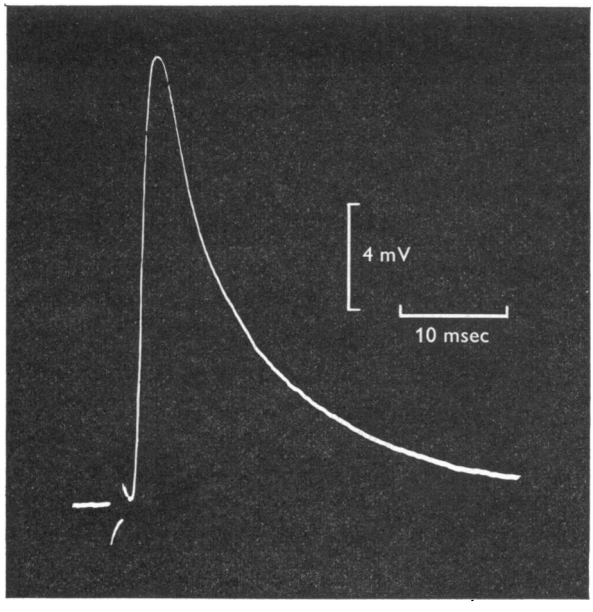
In addition to this unitary release from newly regenerated nerve terminals, we occasionally evoked a sudden large response (Text-fig. 7), which in some instances exceeded the muscle fibre threshold, and looked



Text-fig. 6. Amplitude histogram of unit potentials evoked by focal stimulation of axon terminal at a non-transmitting end-plate (bottom), and spontaneous min. e.p.p.s recorded at the same junction (top). Stimulus frequency, $\frac{1}{2}$ sec. Release evoked by 289 of 1780 stimuli. Ringer solution contained 6 mM calcium, 10^{-6} g tetrodotoxin/ml. Resting potential, 90 mV. Fifteen days after crushing the nerve.

like a normal end-plate potential. Responses such as these were never evoked in the presence of tetrodotoxin. When focal stimulation experiments were first performed on normal frog neuromuscular junctions (Katz & Miledi, 1967) graded, quantal, responses could only be obtained when tetrodotoxin was added to the bath. Without tetrodotoxin, stimulation evoked a sudden large e.p.p. with contraction of a group of muscle fibres due to the initiation of an action potential in the axon terminal which propagated antidromically. It seems probable that the occasional large

responses elicited at the reinnervated end-plates likewise resulted from a local or propagated response in the nerve terminal which caused transmitter release along a greater portion of its length.



Text-fig. 7. Potential evoked at non-transmitting end-plate by focal extracellular stimulation of axon terminal. Subsequent response elicited an action potential in the muscle fibre. Resting potential 85 mV. Fourteen days after crushing the nerve. Ringer solution without neostigmine.

Further support for the hypothesis that failure of nerve-terminal invasion underlies the 'non-transmitting' phase of reinnervation, came from the observation of one fibre which gave no response to a single stimulus to the nerve trunk, but which repeatedly gave an e.p.p. in response to a pair of stimuli 4 msec apart. Presumably in this case a single impulse failed to propagate into the axon terminal but it changed the excitability in the blocked region such that a second impulse would invade the terminal if it followed soon enough after the first. This supposition was strengthened by recording extracellularly from the nerve just proximal to the end-plate. In that situation a single axon spike was recorded in response to the paired stimuli, whereas none was seen with a single stimulus.

In another muscle, a group of three fibres was found which originally had been innervated by one axon (the old myelin is still sufficiently intact after 2 weeks to be seen). These muscle fibres appeared under the micro-

scope to be reinnervated and also had relatively high min. e.p.p. frequencies, but none responded to stimulation of the nerve trunk. By probing in the old myelin with an extracellular electrode, while stimulating the nerve trunk, we were able to record an extracellular nerve spike within three internodes of the end of the myelin. In this case the failure of impulse propagation must have occurred somewhere within the 100 μm between the point of the extracellular recording and the beginning of the end-plates.

All the above findings lend strong support to the hypothesis that in non-transmitting reinnervated junctions the failure of nerve trunk stimulation to evoke an end-plate potential is due to blockage of action potential propagation proximal to the neuromuscular junction.

Reinnervation of the rat diaphragm

It was of interest to know whether the 'non-transmitting' stage of reinnervation is peculiar to frog muscle or whether it occurs also during reinnervation of mammalian skeletal muscle. Therefore, some experiments were made on rat diaphragms which were undergoing reinnervation.

After crushing the phrenic nerve within the thorax, 5–10 mm from the nearest end-plates, restoration of transmission in the hemidiaphragm first occurs by about the fifth day. Examining the muscle fibres during the early stages of reinnervation we again found that some fibres were innervated but failed to transmit impulses or to give end-plate potentials in response to phrenic nerve stimulation. Since in the rat diaphragm spontaneous min. e.p.p.s are virtually absent during the first few weeks after degeneration of the motor nerve (Miledi & Slater, 1968), the min. e.p.p.s observed in these non-transmitting junctions were a good indication of re-establishment of neuromuscular contact. We did not examine the stages of reinnervation extensively, but it seems that the relative number of fibres in the non-transmitting state was smaller than in the frog. This lower proportion may result because the time interval between re-establishment of synaptic contact and resumption of transmission is shorter in mammals, leading to a lower chance of finding fibres in the non-transmitting condition. Upon restoration of transmission, most fibres had only one presynaptic input.

DISCUSSION

Following a cut or crush of the nerve bundle to a muscle, the motor axons grow back into the old end-plate regions and re-establish synaptic contact with the muscle fibres. We have presented evidence that at an early stage of regeneration the axons are in contact with muscle fibres but show no synaptic transmission because nerve impulses do not invade the newly formed terminals. It is clear that some nerve terminals in this non-

transmitting stage are capable of generating action potentials, as indicated by the focal stimulation experiments and also by the fact that a transmitted impulse can be set up by the second of a pair of stimuli (cf. p. 566). However, the possibility still remains that the mechanism for generating action potentials may not be fully developed in the newly formed membrane of regenerating axon terminals.

We have shown that the rate of quantal release from these non-transmitting terminals is influenced by the same procedures which are known to accelerate the min. e.p.p. frequency at normal end-plates, e.g. increased concentrations of potassium and micromolar concentrations of lanthanum in the bathing fluid. It was already known that the min. e.p.p.s at non-transmitting junctions are also accelerated by increasing the osmolarity (Miledi, 1960). These observations leave little doubt that the newly formed axon terminals contain a supply of releasable transmitter.

Earlier work has shown that regenerated junctions at this 'non-transmitting' stage may also exert some 'trophic' effects on the muscle fibre. For instance, the ACh sensitivity of the fibre begins to resume its normal pattern at this intermediate stage (Miledi, 1960; Bennett, Pettigrew & Taylor, 1973). Likewise the non-transmitting terminal can influence the pattern of contracture evoked by KCl or ACh in frog slow muscle fibres (Elul, Miledi & Stefani, 1968).

In most of the experiments with focal stimulation of nerve terminals the release evoked at a given point failed after ten to thirty responses. This might be thought to arise from a limitation in the amount of transmitter available in the new axon terminals, but in other instances the response was well sustained for hundreds of impulses, as illustrated in Text-fig. 6. Furthermore, when lanthanum was applied to terminals at this stage there was an increase in the min. e.p.p. frequency which lasted for many minutes (no attempt was made to estimate the amount of transmitter or the total number of packets released, as compared with normal terminals). It seems most likely, therefore, that the early failure with focal stimulation usually seen was due to some local damage of the new axon by the stimulating electrode. Alternatively, there may be a greater susceptibility to 'fatigue' of the release mechanism in immature axon terminals.

An interesting possibility is that preceding the non-transmitting stage there may be an even earlier phase when the axon terminal has re-established contact with the muscle but does not release any transmitter spontaneously. This could not be detected with the methods used here since we relied primarily on the characteristics of the spontaneous min. e.p.p.s to predict whether or not an end-plate was reinnervated. In a few cases it seemed possible, judging from microscopic observation of the

living tissue, that a nerve was present even though the physiological indications were negative (for example, the few cases mentioned in which application of lanthanum produced no increase in min. e.p.p. frequency of end-plates thought to be reinnervated). To examine such a possibility properly would require a detailed investigation combining physiological with microscopic studies.

There are several detailed questions in regard to the non-transmitting phase of reinnervation, which cannot yet be answered. One is: how long does any one junction remain in this intermediate condition? Another, do all regenerating terminals go through such a stage? If this is the case, then probably each junction remains in it for only a relatively short period, a day or two for the frog and perhaps even less for the rat, for in any one muscle after denervation we found only a few fibres in this state.

It is possible that this condition of imperfect invasion may be a common situation during the development of synaptic contacts, at least at neuromuscular junctions *in vivo*. Not only does it occur in frog and rat, but it has recently been noted during the reinnervation of adult chicken muscle (Bennett *et al.* 1973). In addition, such a phase has been observed during the formation of synapses on newly developed muscle fibres in regenerating salamander limb (M. J. Dennis, unpublished observations). In the latter case it seems that the failure of transmission may again be due to conduction block in the axon since in some instances a double stimulus to the nerve trunk will elicit an e.p.p. when a single one will not, like the situation observed above. The cause of conduction block is unknown. Among possible explanations are excessive axonal branching, and incomplete development of the action potential mechanism. It seems likely that a similar conduction failure may occur in some neuromuscular diseases.

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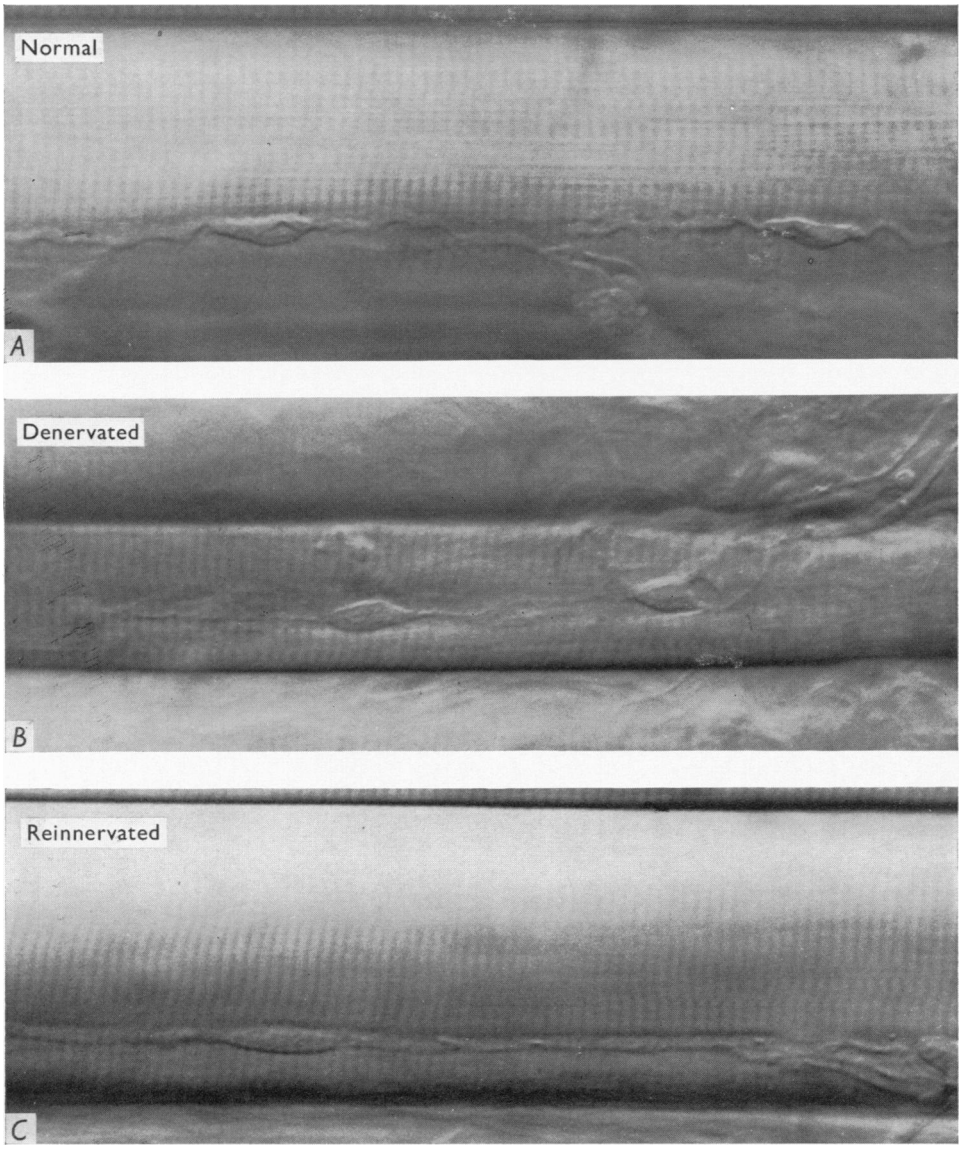
EXPLANATION OF PLATE

Nomarski interference-contrast micrographs of end-plate regions in cutaneous pectoris muscle fibres. *A*, from normal muscle. *B*, from muscle 12 days after denervation. *C*, from reinnervated muscle 20 days after crushing the nerve. Note the characteristic tubular appearance of the axon terminal in the normal and reinnervated end-plates. Schwann-cell nuclei are visible in all end-plates.


Normal
A
Denervated
B
Reinnervated
C