Motor Nerve Terminals As the Site of Initial Functional Changes After Denervation

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ABSTRACT For the cat soleus nerve–muscle system, motor nerve section 48 hr prior to *in situ* experiment causes certain characteristic transmission losses. Responses to repetitive stimulation are sharply altered: The capacity to transmit iterative stimulation is severely reduced; post-tetanic potentiation and the post-tetanic repetition of soleus nerve terminals responsible for it are also greatly impaired; a phenomenon of post-tetanic depression was frequently observed. However, function of the extramuscular axons appears normal and single impulse transmission is usually not seriously affected. The loss of reactivity to repetitive stimulation has been traced to soleus motor nerve terminals. In view of these data and the known absence of denervation hypersensitivity at this time, the earliest functional failure may be said to occur in the unmyelinated terminals. This subacutely denervated preparation therefore offers a simple means of evaluating motor nerve terminal responsiveness.

INTRODUCTION

Studies of denervational changes in neuromuscular function during the first 72 hr after nerve section, have shown that transmission fails at a time when axonal function is intact (5, 9, 17, 22). Some of these early workers suggested but did not demonstrate that motor nerve terminals or end plates are first implicated in the degenerative process (5, 9, 22). In the cat, the absence of denervation hypersensitivity 48 hr after nerve section infers that the post-junctional membrane is not affected at a time when initial transmission loss appears (2, 9, 12, 14, 15). More recently Birks et al. (2) sought to disclose a primary involvement of motor nerve terminals by recording miniature end plate potential (m.e.p.p.) activity in the frog at short intervals after motor nerve section. However, the result was not conclusive, since the pattern of the post-junctionally recorded m.e.p.p.'s was unchanged up to their disappearance on transmission failure.

The present report describes experiments in a cat nerve-muscle preparation *in situ* 48 hr after denervation. The experiments disclose, though pre-junctional recording, that unmyelinated mammalian motor nerve terminals

undergo a clear functional loss prior to their eventual failure to transmit. The results establish that motor nerve terminals are the site of the earliest functional damage following nerve injury.

METHOD

Denervation Cats were anesthetized with pentobarbital sodium, 30 mg/kg intravenously. Unilateral nerve section was performed aseptically mainly at 48 hr prior to experiment (subacute denervation). Depending on the purpose of the experiment, the denervation of soleus muscle was performed by either sectioning the sciatic nerve or severing ventral roots. When only the contractile response of the muscle was to be measured, the sciatic nerve was cut at its point of exit through the sciatic notch. When it was necessary to have the ventral roots remain as part of the cut peripheral nerve, a dorsal laminectomy was performed to expose the lumbar spinal cord and both the dorsal and ventral roots at S1 and L7 were sectioned close to the cord. The roots were repositioned, the dura closed, and the animal allowed to recover.

Experimental Procedures All experiments were performed under α-chloralose anesthesia, 80 mg/kg intravenously. The methods used were essentially those described in previous publications (16, 20, 21). The principal difference was that both the acutely and subacutely denervated legs were simultaneously prepared so that the acutely denervated muscle[1] could serve as a control. Fig. 1 schematizes the arrangement as it was used for the two principal procedures (A and B).

A. *Contractile Responses* The sciatic nerve of the control leg was sectioned at the hip. In each leg, the popliteal fossa was dissected to expose the soleus muscle and its innervation. To record isometrically, the legs were fixed in parallel in the myograph by bone pins and the soleus tendons were each attached via thin steel rods to strain gauges. The medial skin edges and fascia of the two legs were sewn together and the lateral skin edges retracted to form a basin which was filled with paraffin oil. The oil was equilibrated with 95% O_2–5% CO_2 and its temperature maintained at 37°C. The soleus nerves were placed on stimulating electrodes. Tension recordings were made simultaneously from the acutely and subacutely denervated muscles by connecting the strain gauge outputs to the carrier amplifiers of a two channel Texas Oscilloriter (Texas Instruments, Inc., Houston, Tex.).

Direct muscle stimulation was accomplished with a pair of needle electrodes inserted along the muscle length. After blocking neuromuscular transmission with intravenous curare, a supramaximal stimulus was determined.

B. *Motor Nerve Terminal Activity* The legs were prepared as above, except that the sciatic nerve of the control leg was *not* sectioned at the hip. The prior laminectomy site was reopened to expose the lumbar cord. Ventral roots S1 and L7 of the control side were sectioned close to the cord; on the subacutely denervated side, the previously cut ventral roots were recovered. The roots on each side were teased to locate filaments containing one active soleus axon for electrical recording (see Fig. 1). The cord

[1] For simplicity, the acutely denervated or control nerve-muscle system is occasionally referred to as the normal.

was covered with oil continuously bubbled with 95% O_2–5% CO_2. The oil temperature was held at 37°C.

Stimulation To evoke either contractile responses or post-tetanic repetition (PTR) (cf. Fig. 1), supramaximal single stimuli were simultaneously applied from a Grass S-8 stimulator to the acutely and subacutely disconnected soleus nerves. Single

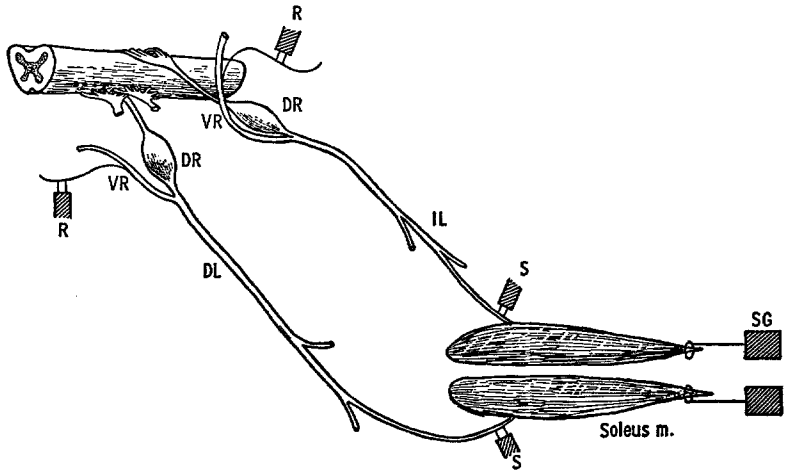

FIGURE 1. Schemes for recording isometric twitch tension and for recording antidromic neural activity. *IL* and *DL* represent acutely and 48 hr denervated legs, respectively. In *DL*, the nerve sections described below were made 48–54 hr prior to experiment. In *IL*, these sections were made acutely. *Twitch*, stimuli were applied to the peripheral nerves with electrodes, *S*, the soleus tendons were connected to strain gauges, *SG*. In this method, the peripheral nerves were cut proximal to the electrodes. *Antidromic neural activity*, stimuli were applied to the peripheral nerves with electrodes, *S*. Electrodes, *R*, recorded from ventral root (*VR*) filaments bilaterally, each containing a single active motor axon. The ventral roots were disconnected from the spinal cord proximal to the recording electrodes.

stimuli were square wave pulses of 0.1 msec duration spaced at 0.4 cps. For tetanic conditioning of motor nerve terminals, 10 sec trains of 20–400 cps were applied to the nerve.

Peripheral Nerve Action Potential Recording electrodes were placed 5 cm centrally to stimulating electrodes located on the soleus nerves. Monophasic action potentials were recorded. The intensity, duration, and frequency of the stimuli were the same as used for production of the maximal isometric twitch response.

RESULTS

Twitch Response to Indirectly Applied Stimulation In the 48 hr denervated muscle, single supramaximal stimulation of the soleus nerve always caused some contractile response. Compare the pre-tetanic twitches in Fig. 2. Assortment of the 48 hr denervated twitch responses into three groups disclosed that

approximately one-fourth are between 0 and 33% of the control; approximately another one-fourth are between 33 and 66% of the control; slightly more than one-half are betwen 66 and 100% of the control. Therefore 48 hr after denervation, a considerable fraction of twitch response remains. In fact, in 12 of the 46 experiments, transmission in the subacutely denervated system was 90% or more of the control.

Beyond the 48 hr period transmission fails rapidly with time, as others have noted (9, 14). In some instances twitch declined during the course of the ex-

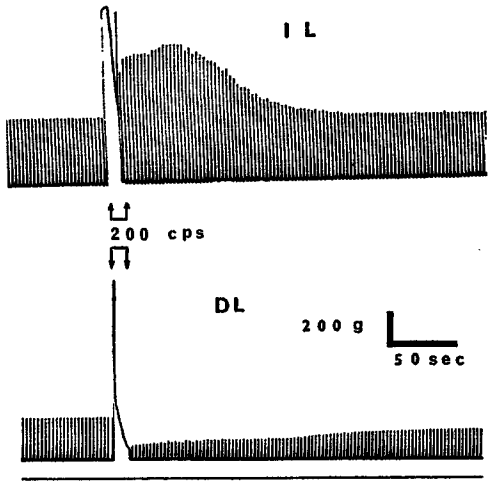

FIGURE 2. Comparison of post-tetanic phenomena in acutely denervated (*IL*) and 48 hr denervated (*DL*) soleus muscles of the same cat. The soleus nerves were stimulated supramaximally once every 2.5 sec. At the arrows, indirect tetanic stimulation of 200 cps was applied for 10 sec. PTP occurs only in *IL*; a post-tetanic depression appears in *DL*.

periment (2–4 hr). That this was not due to deterioration of the preparation was supported in each case by the continued control level response of the normal muscle. In thirteen 60 hr experiments, the average twitch tension was down to 9% of the control and many (8 of 13) did not respond to nerve stimulation. By 72 hr response to nerve stimulation was absent.

To insure the validity of the comparisons between the subacutely and acutely denervated soleus muscles of opposite legs in the same animal, three preparations were made in which maximal twitch tensions were recorded from acutely denervated muscles of both legs. In each experiment the ratio of the twitch tensions of both muscles approximated unity and hence there were no significant differences.

Twitch Response to Directly Applied Stimuli When equal supramaximal stimuli were applied directly to the 48 hr denervated and normal muscles of

the curare-paralyzed animal, there were no differences between the amplitudes and shapes of the twitch responses up to 72 hr after denervation.

Compound Nerve Action Potential In view of the twitch tension differences between the subacutely denervated and normal muscles, it was important to know whether the distal sciatic nerve segments were functionally comparable. The compound action potentials of soleus nerves, disconnected acutely or 48 hr previously showed similar amplitudes and time courses. Moreover, thresh-

TABLE I

COMPARISON OF ACUTELY AND 48 HR DENERVATED MUSCLES FOR THE MAINTENANCE OF MUSCLE TENSION IN RESPONSE TO DIFFERENT FREQUENCIES OF INDIRECT STIMULATION

The data were obtained quantally. If tension was not fully maintained, it was counted as a failure. The columns on the right present for 46 experiments the percentage incidences in which tetanic tension was maintained by the acutely and 48 hr denervated muscles.

	Maintaining tetanus	
Frequency of tetanus	Acutely denervated	48 hr denervated
cps/10 sec	%	%
20	100.0	93.3
40	100.0	76.5
100	100.0	47.1
200	73.3	17.6
300	15.4	0.0
400	0.0	0.0

olds, conduction velocities, and frequency responses of the nerves were the same. Hence, the decline in responsiveness of the 48 hr denervated muscle to indirect nerve stimuli probably has its origin more distally, as others have concluded (5, 9, 17, 22). After 48 hr amplitude of the compound action potential and conduction velocity declined rapidly.

Response to Repetitive Nerve Stimulation Repetitive nerve stimulation regularly disclosed important differences between the 48 hr denervated and the normal systems. Prominent was a reduced maintenance of tetanic tension as the frequency of nerve stimulation increased. Comparison of the tetanic responses depicted in Fig. 2 illustrates the quick failure of tetanus maintenance in the 48 hr denervated preparation, as contrasted with the well-sustained response in the normal nerve-muscle system.

For 17 cats denervated 48 hr previously, tension maintenance in response to different frequencies of stimulation is shown in Table I. The tabulated figures indicate that the normal muscle in every instance can hold a complete tetanus in response to nerve stimulation as high as 100 cps. In sharp contrast, only

47% of the subacutely denervated muscles sustain a response at 100 cps and, in fact, even when the frequency was as low as 40 cps, only 77% maintained tension. Above 100 cps, tetanus maintenance falls off for both the normal and subacutely denervated muscles, but the failure of the latter is much more striking.

In view of these differences, it is important to recall (see above) that the distal sciatic nerve segment of the 48 hr denervated preparation follows a fre-

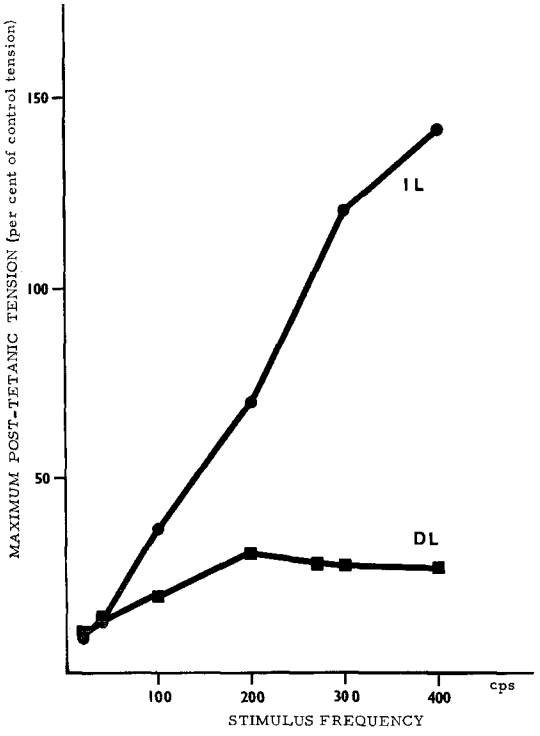

FIGURE 3. Effect of 48 hr denervation on PTP of indirectly stimulated cat soleus muscle. Ordinate, maximum PTP is expressed as per cent increase above the pre-tetanic twitch tension. *IL* and *DL* indicate respectively, acutely and 48 hr denervated muscles. Each point represents the average of 17 experiments. Abscissa, frequency of the indirect 10 sec conditioning stimulus.

quency of 400 cps as well as does the normal nerve. Hence, the tetanic deficit 48 hr after denervation cannot be attributed to failure of extramuscular axons.

Post-Tetanic Potentiation (PTP) To test the possibility of motor nerve terminal damage in subacute denervation, advantage was taken of the finding of Standaert (20, 21) that PTP of indirectly stimulated cat soleus muscle develops almost entirely from a post-tetanic repetition of the motor nerve terminals. In the case of the 48 hr denervated preparation, it was found that PTP of soleus muscle indirectly stimulated is grossly reduced or lost. Thus in 10 of 17 experiments, PTP *did not* occur after a 10 sec conditioning stimulus of 200 cps. For the control muscles of the contralateral leg, PTP developed in all but 2 of 17 instances. In 3 of 7 48 hr denervated muscles, in which PTP did occur after 200 cps conditioning, the magnitude was considerably reduced. An

extreme example of the effect of 48 hr denervation on PTP development is shown in Fig. 2. In this experiment, the acutely denervated muscle exhibited a typical sharply evolved PTP with a peak tension at 205% of the pre-tetanic twitch; the 48 hr denervated structure showed not only a total failure of PTP but also a severe post-tetanic depression (PTD). If a small PTP did appear in the subacutely denervated structure, its time course consisted of a delayed onset, a slow rate of rise, and a slow recovery.

For the 17 experiments with 48 hr denervated muscle, the average PTP loss is graphically displayed in Fig. 3. The higher the frequency of the conditioning tetanus, the greater is the failure of PTP; this is evident from the disparity between curves for the normal and 48 hr denervated muscles.

Post-Tetanic Depression (PTD) PTD occurs frequently in the 48 hr denervated preparation. Fig. 2 discloses an impressive example of this, especially in comparison with the post-tetanic potentiation of the normal muscle. The peak PTD was only 33% of the pre-tetanic twitch height.

For 17 experiments with short-term denervated muscles, the incidences of PTD following various high frequencies of stimulation were: 18% at 100 cps, 65% at 200 cps, 71% at 300 cps, and 85% at 400 cps. The higher the frequency of the tetanus the greater the likelihood of PTD. A characteristic aspect of PTD is that in all instances recovery was either slow, incomplete, or absent.

Post-Tetanic Repetition (PTR) To confirm the probable motor nerve terminal damage indicated by the PTP experiments, it was necessary to demonstrate directly that PTP loss is due to deficient PTR generated by the terminals. The PTR experiments presented a formidable problem. It was necessary to section the motor nerve at the ventral root level but yet have the severed axons functional approximately 48 hr later. In this way the ventral root axons could be used to monitor motor nerve terminal activity. The scheme of these experiments is detailed in Fig. 1.

Six such experiments enabled the sampling of 33 axons which had been sectioned between 48 and 54 hr previously. In each case, these axons were paired with an equal number of contralateral motor axons acutely disconnected. Every axon used was proved to be in satisfactory condition by demonstrating its capacity to conduct stimuli up to 400 cps.

In 3 of 4 experiments, performed 48 or 52 hr after nerve section, PTR was reduced to 74, 41, and 10% of the control. In one of the 48 hr experiments, the PTR from both the control and subacutely denervated terminals was equally good.

Although the adverse effect of subacute denervation on PTR was clear, it was also apparent that the increased length of axons in these experiments (approximately 5–6 cm as opposed to the usual experiments in which the

nerve was cut at the hip) had probably attenuated the effect of the denervation. A direct relationship between the length of the distal axon and the persistence of synaptic function after denervation has been described (5, 10, 18). To compensate for this, two PTR experiments were performed at 54 hr after ventral root section. This period was based on the estimate of Slater (18) in which each centimeter of axon represents about 40 min in the progression of the degenerative process. In these experiments, the functional integrity of the

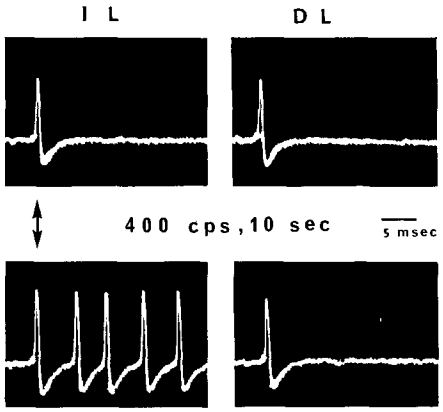

FIGURE 4. Simultaneous comparison of post-tetanic repetition (PTR) in single motor axons from acutely (*IL*, left column) and 48 hr denervated (*DL*, right column) soleus muscles of the same cat. Single stimuli were delivered to the soleus nerves at a frequency of 0.4 cps; recordings were obtained from single axons of the right (*DL*) and left (*IL*) *L7* ventral roots. For each axon the upper tracings show the evoked antidromic action potential. After stimulation of 400 cps for 10 sec, the left lower tracing shows the repetitive discharge generated by the motor nerve terminal of the *IL* axon in response to a single stimulus. The right lower tracing shows no PTR.

myelinated axons from which recordings were made was assured by the normalcy of their response to high frequency stimulation.

In one 54 hr experiment, 16 denervated and 16 control axons were compared; in the other, 7 axon pairs were studied. In both experiments the results were dramatic; PTR occurred in 21 of 23 control axons; it did not occur in any of the 23 short-term denervated axons. Also, in accord with the PTR failure, PTP was absent in the subacutely denervated muscles.

Fig. 4 illustrates PTR loss in one of the 54 hr experiments. Prior to tetanic conditioning the single stimuli led to single evoked antidromic responses in both acutely and 54 hr sectioned nerves. Following conditioning with 400 cps for 10 sec, single evoked responses were followed by as many as four PTR's in the case of the acutely sectioned nerve, while no PTR appeared in the nerves cut 54 hr earlier.

The results emphatically disclose that the subacutely denervated terminal's

capacity to generate PTR is reduced or lost, even though it may retain the ability to transmit.

DISCUSSION

Conclusive support for motor nerve terminals as the sites at which functional loss first appears following motor nerve section, comes from those experiments concerned with post-tetanic events. Since PTP of soleus muscle has been shown to be a direct consequence of PTR of motor nerve terminals (20, 21), the loss or severe reduction of PTR, and hence PTP, that occurs 48 hr after denervation reliably signals motor nerve terminal dysfunction.

In contrast, signs of transmission failure indicated by either loss of twitch height or reduction in end plate potential (e.p.p.) amplitude would not be diagnostic of the point of injury. The sensitive and specific nature of soleus nerve PTR establishes that the first functional defect occurring in the distal axon after nerve section is in the terminal. This finding is an explicit confirmation of the more general conclusion of previous investigators who decided by exclusion that the junctional region is the point of initial breakdown (5, 9, 22). The demonstration of functional failure in terminals conforms with morphologic findings in the mammal and the frog, wherein the earliest abnormalities involve the terminals, not axons or post-junctional membranes (2, 13, 19).

The PTD, subtly but unequivocally, reflects pre-junctional failure. On inspection alone, PTD or twitch would appear to represent either a pre- or post-junctional deficit. But the nerve terminal lesion is revealed by the data showing that soleus PTD, like PTP in that muscle, is progressively increased as a function of the frequency of the indirect conditioning stimulus (cf. Results). In this respect, it is necessary to appreciate that transmission of frequencies above 100 cps declines sharply (Table I), especially in the denervated system. Consequently, the increasing effectiveness of frequencies up to 400 cps in both the normal (see also reference 21) and the subacutely denervated junctions in evoking PTP or PTD, respectively, discloses motor nerve terminals as the responsive structures. Presumably PTD shows that after intense depolarization of subacutely denervated terminals, repolarization is either very slow or absent.

A persistent post-tetanic depolarization of the 48 hr denervated terminals would also explain their failure to generate PTR. The post-tetanic depolarization, unlike the normally occurring post-tetanic hyperpolarization (20), would decrease rather than increase the amplitude of the nerve terminal spike potential. Consequently, the usual post-tetanic enlargement of negative after-potential would not occur and so the generator for PTR would be absent.

Defective motor nerve terminal recovery is probably also manifested by the loss in maintenance of an indirect tetanus. For normal mammalian motor

nerve terminals, the present results, as well as those of Krnjevic and Miledi (8) show that prejunctional failure occurs during stimulation in excess of 100–200 cps. Thus in the early stages of denervation, it appears that this kind of pre-junctional block would be greatly exaggerated because of the damaged terminals' poor recovery capacity.

In view of the evidence, the loss of twitch height at 48 hr can be attributed to a complete failure of some of the terminals. Present as well as other findings (5, 9, 17, 22) amply demonstrate normal excitability characteristics for myelinated axons at the 48 hr period. With regard to muscle, direct stimulation shows no difference in response between the 48 hr and acutely denervated muscle. More important, the experiments suggest that the post-junctional response to transmitter activity is not altered in the 48 hr period, since twitch tension can be at or near the control level, although nerve terminal function (PTP and PTR) is demonstrably impaired. The implication of an unaltered post-junctional membrane is supported by the finding (2) that e.p.p.'s recorded intracellularly in denervated frog muscle do not show any deviation from normal up to their disappearance on transmission failure.

Recognition of early motor nerve terminal failure has interesting implications for neurologic and physiologic aspects of neuromuscular transmission. While the trophic influence of nerve on muscle has long been an intriguing problem, it appears from present data that it is not facetious to state that there is an important "trophic" influence of the neuron on its terminal. Why the distant terminal structure should first exhibit injury is not clear. Most reported axoplasmic flow rates of various cellular constituents (3, 4, 7) are too slow to suggest that deprivation of essential materials is responsible. A few recent reports (1, 11) infer extremely rapid axonal transport of tritiated leucine. But Barondes (1) cautions that any one of several artifacts may account for the seemingly rapid movement. Initial terminal susceptibility might be thought attributable to diameter or unmyelination. However, experience of others (6) indicates that after denervation, small diameter fibers and unmyelinated axons retain function slightly longer than do the larger diameter and the myelinated fibers. For whatever, as yet unknown reasons, the fact remains that the terminal is the most reactive part of the entire axonal structure to main trunk injury.

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