TRANSMITTER RELEASE PROPERTIES ALONG REGENERATED NERVE PROCESSES AT THE FROG NEUROMUSCULAR JUNCTION¹

PAIGE DECINO²

Department of Physiology, UCLA School of Medicine, Los Angeles, California 90024

Abstract

Reinnervation following nerve crush was examined in the frog cutaneous pectoris muscle using histological and electrophysiological techniques. Functional recovery, as monitored by miniature endplate potential (mepp) frequency and quantal content, was slower than morphological recovery. To understand this discrepancy, extracellular recording electrodes were used to determine the amount of transmitter released from localized areas of nerve terminals in both unoperated and experimental muscles. There was no increased tendency over controls for the proximal portion of a regenerated nerve process to release more quanta (spontaneous or nerve evoked) than the distal portion. Moreover, extracellular recording at the growing nerve tip showed that this portion of nerve terminal was capable of transmitter release upon nerve stimulation. These results suggest that the entire length of a regenerating nerve terminal becomes functional at early stages of reinnervation and matures at essentially the same rate.

Reinnervation has long been used as a model for development of the nervous system in addition to being used to investigate the recovery process following nerve injury. To study reinnervation, the peripheral nervous system is often preferred over the central nervous system since cells in the periphery are more easily identified and manipulated. In particular, the frog neuromuscular junction, which has been studied extensively, has a geometry which facilitates studying transmitter release properties from restricted areas of nerve terminal processes as well as correlating structure and function (see Kuno et al., 1971; Grinnell and Herrera, 1980).

Since Miledi (1960) first examined reinnervation in the frog physiologically, additional studies (Dennis and Miledi, 1971, 1974a, b) have contributed to our understanding of functional recovery following nerve crush. They described the sequence of events during reinnervation as follows: After a quiescent period, Schwann cell miniature endplate potentials (mepps) appear. Subsequently, spontaneous nerve release begins, but without concomitant nerve-evoked endplate potentials (epps). This nontransmitting stage of reinnervation is followed by the appearance of epps which become suprathreshold as the synapse matures.

In the course of their investigation on reinnervation, Dennis and Miledi (1974b) suggested that there may be proximal-distal gradients for both spontaneous and nerve-evoked transmitter release along regenerating nerve processes such that the proximal portion releases transmitter more frequently than the distal, presumably less mature, portion. However, it is also possible that the rate-limiting step for functional recovery is the time required for the nerve to completely reinnervate the postsynaptic membrane. The purpose of the present work was to distinguish between these possibilities.

The first step in the present investigation was to correlate structural and functional recovery after nerve crush to determine whether these two aspects of reinnervation proceed at different rates. The results of this investigation show that physiological recovery of synaptic transmission after nerve crush lags behind morphological recovery. The second step entailed studying certain aspects of reinnervation in more detail by recording from small numbers of release zones along regenerating processes to ascertain whether there are gradients in release. These experiments reveal that the discrepancy between structural and functional recovery cannot be explained by differential maturation of the nerve terminal arborization as suggested by Dennis and Miledi (1974b). Rather, it seems that the entire nerve slowly develops, at the same rate, the capability to release more quanta of acetylcholine.

Materials and Methods

Preparation. The thin cutaneous pectoris (cp) muscle from adult *Rana pipiens* (5 to 6.5 cm body length) was used in these experiments. Muscles were denervated by

¹ I wish to thank Paul Brehm, Alan Grinnell, Mike Letinsky, and Katie Morrison-Graham for criticism of the manuscript. I greatly appreciate the assistance given by Mike Letinsky during the course of these experiments. In addition, I thank Ira Kohn, Jose Rodriguez, and Herman Kabe for technical assistance and Bonnie Harkins for preparation of the manuscript. This study was supported by United States Public Health Service Grant NS13470; P. D. was supported by United States Public Health Service Training Grant 5 to 1GM0048.

² Present address: The Salk Institute, Neurobiology Lab, Box 85800, San Diego, CA 92138.

crushing the cp nerve 1 to 2 mm from its entry into the muscle after the animal had been anesthetized with tricaine methanesulfonate (Ayerst). Contralateral muscles were not used as controls because of denervation-induced changes in the unoperated contralateral cp (Rotshenker, 1979). Instead, control cps were studied from similarly sized, unoperated frogs. Frogs were maintained at room temperature (21 to 24°C) and fed live crickets. Experimental muscles were examined electrophysiologically and histologically, 3 to 30 days postoperatively.

Muscles were dissected along with a length of nerve (0.5 to 1.0 cm) or with the entire nerve trunk back to the spinal cord (2 cm). The muscles were pinned out to 120% of their resting length in Sylgard (Dow Corning) resin-lined 60-mm Petri dishes for subsequent electrophysiological experiments (McMahan and Kuffler, 1971). This amount of stretch increased spontaneous release, facilitating measurement of mepp frequency (Hutter and Trautwein, 1956) which was particularly useful in early reinnervated muscles. The gross pattern of the nerve trunk was drawn with a camera lucida at a magnification of about $\times 30$. This muscle map allowed later location of identified endplates. Hoffman modulation contrast optics were employed to visualize living nerve terminals at $\times 400$ magnification during the course of the experiment. An eyepiece micrometer was used to measure distances.

Solutions. Normal frog Ringer's (NFR) solution was used during the dissection and, in some experiments, on miniature endplate potentials (mepps). This solution contained (in millimoles/liter) 110 NaCl, 2 KCl, 1.8 CaCl₂, and 5 HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, Sigma) buffer to give a pH of 7.2 to 7.3. Neostigmine (Roche) was used only occasionally at a concentration of 10^{-6} gm/ml because it causes a decrease in mepp frequency (Duncan and Publicover, 1979). In order to increase mepp frequency for focal recordings, particularly in the younger reinnervated muscles, the osmolarity of the NFR was often increased by the addition of 50 mM sucrose (Furshpan, 1956). Muscle twitches were blocked with frog Ringer's solution of the above composition but with 2 mM MgCl₂ and reduced CaCl₂ (range, 0.3 to 0.45 mM). Quantal contents were determined in NFR containing curare at a concentration of 2 to 5×10^{-6} M (Abbott Laboratories).

Electrophysiology. Intracellular recordings were made with omega dot microelectrodes (resistance, 50 to 100 megohms). Mepp amplitude was corrected to an arbitrary resting potential (E_r) of -90 mV (amplitudes corrected for nonlinear summation by a factor of $75/(-15 - E_r)$, assuming a reversal potential of -15 mV) and only those fibers with E_r values of at least -80 mV were used. When quantal size (calculated as average mepp amplitude $\div$ input resistance, R_{in}) was to be measured, two microelectrodes separated by approximately 25 μ m were employed to determine R_{in} by square pulse analysis. Experiments were done at 18°C.

Nerve-evoked transmitter release was examined at a stimulation frequency of 0.5 Hz using both intracellular and extracellular recording microelectrodes. When the time course of functional recovery was being followed, quantal content of the curare-blocked nerve terminal was determined by the coefficient of variation method from approximately 100 intracellularly recorded epps (Martin,

1960). In low calcium, high magnesium Ringer's solution, quantal content was determined from mean epp amplitude divided by mean mepp amplitude (Martin, 1960). In both cases, epp amplitudes were corrected to an E_r of -90 mV before calculating quantal content.

In order to test for gradients along a nerve terminal branch for spontaneous and evoked events, focal, extracellular recordings were made simultaneously with intracellular recordings. Focal recordings were made using either broken tip microelectrodes or electrodes pulled on a microforge with outer diameters of approximately 2 μ m and filled with NFR. By placing two extracellular electrodes different distances apart, it was determined that half of the spontaneous events recorded at one position were also recorded at the second electrode when the separation was 10 μ m, thus defining a field size of 20 μ m. For practical reasons, the field size could not be checked by this method every time. Therefore, as a routine check of whether the field sizes were approximately equal, the average extracellular event size was determined for each recording site. Field sizes at two positions (separated by at least 30 μ m) were deemed equal if mean extracellularly recorded amplitudes at each position varied less than 20% with respect to the median of the two values. Even when field size at one point was larger than at another, information sometimes still could be obtained. If, for example, mepp frequency was greater at one position with a smaller field size relative to another location, the qualitative difference in mepp frequency was considered real since a larger field size at the first position would likely only increase the incidence of mepps and not change the relative ranking of mepp frequencies from the two positions.

Monitoring field size was done because, as the electrode is pulled away from the release site, both the individual and average focally recorded events become smaller (Wernig, 1976). The above method of determining field size assumes that the average extracellularly recorded events from areas of like size will be the same. Support for this assumption comes from results on focally recorded mepp amplitudes which showed that there are no consistent differences in quantal size along normal and reinnervated nerve terminal branches (see "Results"). Hence, since quantal size is the same, the average amplitude of extracellularly recorded events can be taken as a measure of field size.

In addition to field size, damage to the nerve terminal by the extracellular electrode must be monitored carefully if estimates of focal frequency of release are to be made. Therefore, intracellular mepp frequency was monitored before and after placement of the extracellular electrode(s). Changes of more than 25% from the initial rate (the range of variability in mepp frequency determined at different times from experimental cps) were taken as an indication of injury and the data were not used. For evoked release, quantal content (m) of the entire magnesium-blocked terminal was determined first and then monitored at the same time as the extracellularly recorded evoked responses. If m changed by more than 20% (the normal variability range in low calcium) for the whole terminal, then the data were not included.

In determining the presence of gradients along a terminal process, differences in focal mepp amplitude, mepp frequency, and probability of evoked release were rated

in the following manner: Positive gradients were those in which the measured parameter was greater at the proximal position where the nerve enters the endplate region, than distally (see Fig. 1). Negative gradients were those where the measured parameter was greater distally than proximally. Where there were no differences or values at more than two recording sites proved inconsistent, a zero rating was given.

Determination of differences for evoked and spontaneous events between two sites was done as follows: For mepp amplitudes, average sizes were compared using the Student's *t* test with a significance level of 0.025. Mepp frequencies were classed as different if they varied by more than 30% (5% greater than the normal variability range, see above) from the frequency obtained from the most proximally recorded site along a terminal branch when the field sizes were comparable. The probability of evoked release from a focally recorded extracellular site was calculated as the number of recorded focal events divided by the total number of trials. When field sizes were approximately the same, a difference of 25% (5% greater than the normal variability range, see above) from the proximal probability of evoked release was taken as significant. In some cases, a terminal branch was only checked for the presence of evoked release at a number of points without attempting to quantitate the amount of release.

When correlating structure and function, the endplate, as observed through Hoffman optics, was sketched and then located on the previously drawn muscle map. This method allowed identification of these physiologically characterized terminals after staining of the endplate. Following measurement of terminal length (see below), mepp frequency and quantal content were normalized to the 100 μ m length of terminal. When focal recordings were to be made, the fiber first was photographed with a Polaroid camera in order to record accurately the position of the extracellular electrodes. These fibers were subsequently located as above.

Histology. After the electrophysiology of reinnervated and control muscles was completed, the neuromuscular junctions were stained by the combined nitro blue tetrazolium (NBT; Sigma)/acetylcholinesterase (AChE) method (Letinsky and DeCino, 1980; Karnovsky and Roots, 1964) as follows: After a 10 min prefixation in 2.5% glutaraldehyde, the whole muscle was stained with NBT (1 mg/ml) and phenazine methosulfate (0.1 mg/ml, Sigma) in 2.5% glutaraldehyde for 2 to 3 min. The AChE was stained next by the Karnovsky and Roots (1964) technique. Postfixation was first done in 1% OsO_4 (Pelco) for 0.5 to 1 min followed by 2% acrolein (Matheson, Coleman & Bell) for 30 min and then the whole muscle was mounted between coverslips in Hydramount (Gurr). With this procedure, the nerve terminals stained blue and were outlined by the brown cholinesterase reaction product (see Fig. 1, Letinsky and DeCino, 1980). Identified muscle fibers from which recordings were made previously were located and the terminal arborization and the subsynaptic AChE were drawn with a camera lucida using $\times 40$ or $\times 63$ Zeiss planapo objectives. Measurements of overall terminal length and percentage of filled gutter were made in the following manner. Ter-

минаl length was calculated as the total length of NBT-stained terminal processes overlying postsynaptic AChE (Fig. 1). The percentage of filled gutter was calculated as terminal length ($\times 100$) divided by the total length of AChE-stained membrane (Fig. 1). The number of axons innervating the fiber was noted as well as the presence of overgrowth (NBT-stained nerve processes growing beyond the confinement of the postsynaptic membrane, see Fig. 1, *arrowheads*). Sarcomere spacing was measured in order to normalize terminal length to a sarcomere spacing of 2.2 μ m to allow comparisons between fibers. Focal recording positions could be located accurately on the drawing after comparison with Polaroid micrographs of the living terminal processes.

Results

Recovery of regenerated nerve terminals after nerve crush

The initial portion of this study followed reinnervation both morphologically and electrophysiologically to determine if normal neurotransmitter release returned with the same time course as morphological recovery.

Morphological recovery. The pattern of reinnervation at the endplate region could be followed by employing the combined NBT/AChE staining technique (Letinsky and DeCino, 1980). At about 3 days after nerve crush, only fragmented, degenerating nerve remaining at the synaptic site was stained with NBT (Fig. 2). The type of degenerating terminal processes illustrated in Figure 2 was not seen beyond 5 days after crushing. When the nerve began to reinnervate the original endplate (Letinsky et al., 1976) at about 5 to 6 days, processes which were often quite thin (less than 0.5 μ m) began to grow

Figure 1. Calculation of morphological parameters. *Panel A* shows a reinnervated muscle fiber (22 days postcrush) stained using the NBT/AChE technique. To better visualize the endplate region, a camera lucida drawing was made (*B*). The regenerating nerve grows back to the original endplate (indicated by the crossbars denoting the AChE reaction product). The percentage of filled gutter = $x / (x + y)$, where x is the length of nerve overlying AChE (terminal length) and y is the amount of empty gutter. The asterisk indicates a length of empty gutter. *Arrowheads* indicate where the nerve has grown beyond the gutter (overgrowth). There are two axons (*I*) supplying this fiber. The letters *P* and *D* refer to proximal and distal positions along a nerve branch, respectively. *Bar* = 10 μ m.

into the previously denervated postsynaptic gutter (Fig. 3A₁). The camera lucida drawing (Fig. 3A₂) accompanying the 6 day reinnervated endplate in Figure 3A₁ shows more clearly the growing nerve processes in relation to the postsynaptic AChE. Note that, at the *arrows* in Figure 3A₂, the regenerating nerve has only partially grown back into an empty gutter. Only prior to 8 days was it common to see a particular branch of postsynaptic gutter partially reinnervated. As reinnervation progressed further, individual gutters in the postsynaptic membrane became completely filled with the growing nerve which often grew beyond the confines of the original gutter as defined by the AChE reaction product (Fig. 3B). Endplates such as illustrated in Figure 3B were occasionally seen as early as 6 days on the lateral edge of the muscle where the nerve enters. However, by 10 days, all endplates were at least partially reinnervated.

To quantitate morphologically the extent and time course of reinnervation, the percentage of postsynaptic membrane covered by stained nerve terminal processes (i.e., filled gutter) at different times after denervation was determined (Fig. 4, *open squares*). After about 12

Figure 2. Denervated endplate. A 3 day postcrush muscle fiber is illustrated which shows fragments of NBT-stained nerve within the postsynaptic gutter (at *arrows*, and within *brackets* (▲—▲)) Bar = 10 μ m.

Figure 3. Reinnervated muscle fibers. This figure shows the photomicrograph (A₁) and camera lucida drawing (A₂) from a 6 day postcrush cp. Gutters are filled incompletely at sites indicated with *arrows*. *Arrowheads* mark points of overgrowth. There are two inputs (I). Panel B is a montage of a 22 day reinnervated endplate. This endplate has two inputs (*arrowhead*) and overgrowth (*small arrow*) growing between this and a second endplate on the adjacent muscle fiber. At the *large arrow*, there is a nerve process growing into an already innervated gutter from a nearby gutter. Bar = 12 μ m in A₁ and A₂; 10 μ m in B.

Figure 4. Summary of nerve-filled postsynaptic membrane, mepp frequency, and quantal content as a function of time after nerve crush. The percentage of subsynaptic membrane covered by nerve processes is indicated with *open squares* ($n = 23$ to 52 for each point). Mepp frequency/100 μ m of terminal length as a percentage of control levels is represented by *open triangles* ($n = 3$ to 19 for each point). Quantal content/100 μ m of terminal length as a percentage of the control value is represented by *filled circles* ($n = 9$ to 19 for each point). SEM bars were not included for the sake of clarity but were in the range of 2 to 7% for filled gutter, 1 to 11% for mepp frequency, and 8 to 19% for quantal content.

days following nerve crush, extensive reinnervation had occurred; approximately 95% of the subsynaptic membrane was covered with growing nerve terminal processes. There was a wide range in percentages (not shown) of filled gutter at day 6 due to the fact that the nerve enters the lateral edge of the muscle and generally reinnervated endplates closest to that edge first. Thus, the more medial muscle fibers were often still completely denervated at this time.

Functional recovery. Normalized mepp frequency as a percentage of control values at varying times after denervation is shown in Figure 4. Intracellular recordings from visually identified endplates in NFR were maintained for at least 2 min (usually 3 to 5 min) to determine if mepps were present. In cases where mepp frequency was determined to be zero, the fiber was stained to insure that it was innervated. Zero mepp frequency in morphologically reinnervated fibers was only seen at 6 days after nerve crush (7 of 22 cases). Mepps with slow rise times (time to peak > 4 msec) were seen on occasion at 6 to 10 days and were included in the calculation for mepp frequency. No attempt was made to determine if the slower mepps originated from distant endplates. An estimation of Schwann cell mepp frequency was made from one muscle whose nerve had been cut 8 days previously. NBT/AChE staining confirmed that the muscle was completely denervated at the time of the experiment. In this case, the mean mepp frequency from 11 fibers was 0.03/sec. The low frequencies seen during the early stages of reinnervation (e.g., 0.04/sec at 6 days) may be partly due to Schwann cell mepps, but histological evidence showed that the fibers were reinnervated and, in all cases, some mepps with fast rise times (time to peak < 2.5 msec

compared to >5 msec for Schwann cell mepps) were seen. Figure 4 (*open triangles*) shows that normalized mepp frequency only attained 49% of the normal value after 30 days in spite of the fact that the muscle had been reinnervated completely by morphological criteria since day 12.

Positively skewed mepp amplitude histograms (due to large numbers of small mepps) have been reported in reinnervated muscle (Dennis and Miledi, 1974a), developing muscle (Kriebel and Gross, 1974; Bennett and Pettigrew, 1975), and nerve-muscle cultures (Anderson et al., 1979) of amphibians. It was of interest, therefore, to check for the presence or absence of skewedness in mepp amplitude histograms. In general, the amplitude histograms did not appear skewed even at early times after nerve crush (Fig. 5A). However, a few endplates displayed skewed amplitude histograms (Fig. 5C). Since Dennis and Miledi (1974a) used neostigmine and since

synapses on cultured *Xenopus* myotubes have no demonstrable AChE activity (Y. Kidokoro, personal communication), the actual histogram distribution might be obscured by the smaller mepps being lost in the noise. Therefore, neostigmine was added to the Ringer's solution in a few cases. Figure 5, A and B shows mepp amplitude histograms from the same muscle fiber with and without neostigmine. As can be seen, the neostigmine had no effect on the overall shape of the mepp amplitude distribution in this case. In light of these results, it is not surprising, then, that when quantal size (average mepp amplitude $\div R_m$) was calculated, at no time after nerve crush was it smaller than the control value.

The time course of functional recovery as characterized by nerve-evoked transmitter release was investigated also to determine if it followed the same pattern as spontaneous release. Figure 4 shows that quantal content, m , in curarized muscle returns to normal levels by 22 days postcrush. While quantal content seems to recover faster than mepp frequency, it still lags behind morphological recovery.

Intermediate stages of functional recovery characterized by nontransmitting endplates and subthreshold epps (Dennis and Miledi, 1974a, b) were very rarely seen in this species of frogs. In addition, no evidence for a discrepancy in mepp amplitude and unit epp amplitude (Fig. 6) was found. This is consistent with the earlier results showing the absence of smaller than average mepps.

Figure 5. Mepp amplitude histograms. A, Mepp amplitude histogram from the same muscle fiber as in B before treatment with neostigmine. B, After neostigmine treatment. C, Mepp amplitude histogram from untreated fiber. Both muscle fibers from 10 day reinnervated muscle. Black bars on abscissa represent the noise level. Arrows indicate the mean value. In A, $n = 79$; in B, $n = 137$; in C, $n = 157$.

Figure 6. Mepp and epp histograms from an 8 day reinnervated muscle. Panel A shows the histogram of mepp amplitudes ($n = 133$); B is the histogram for epp amplitudes ($n = 195$). Black bars on the abscissa represent the noise level; arrows indicate the mean value. In B, the *open bar* on the ordinate represents the number of failures.

Figure 4 summarizes the functional and morphological recovery following nerve crush and indicates the time difference in these two aspects of reinnervation.

Release properties of localized areas of regenerating nerve processes

Focal recordings from restricted areas of nerve terminal were made to determine if the temporal differences in morphological and functional reinnervation could be explained in terms of the proposed proximal-distal gradients for transmitter release (Dennis and Miledi, 1974b). Mepp amplitude, mepp frequency, and the probability of evoked release from areas of approximately 20 μm (see "Materials and Methods") were calculated at two or more positions along individual nerve terminal branches in both control and experimental preparations (see "Materials and Methods").

Mepp amplitude. Mepps were recorded with intracellular electrodes while monitoring extracellular events at a particular nerve terminal position(s) (Fig. 7A) to determine if quantal size varied along a regenerating nerve terminal process. The intracellularly recorded mepps corresponding to the focal recording site were averaged after correcting for E_r . Overall, there were no systematic differences in intracellular mepp amplitudes recorded at different points in control and reinnervated muscles. This is illustrated in Figure 8 which compares the frequency of occurrence of gradients in mepp size for normal and experimental animals (subdivided into early and late stages). For early reinnervation times (8 to 10 days),

Figure 7. Intracellular and extracellular oscilloscope traces from 8 day postcrush muscles. The top trace in each sweep is the intracellular record (i), the middle trace (e_1) is an inverted extracellular record, and the bottom trace (e_2) is another extracellular trace of normal polarity. A, There are six oscilloscope sweeps recording mepps with two corresponding extracellular mepps in e_1 on the second sweep; for e_2 , there are extracellular mepps in the fourth and sixth sweep. Electrodes were 32 μm apart. B, Three sweeps recording intracellular epps and corresponding extracellular events. In the first sweep, there is a corresponding extracellular event in e_1 ; in the second sweep, there is an extracellular event in e_2 and a small event in e_1 . The extracellular electrodes were 51 μm apart. Calibration: 2 mV (i); 500 μV (e_1 , e_2); 100 msec (A); 2 msec (B).

Figure 8. Percentage of type of gradient for mepp amplitude from normal and reinnervated muscles. Each bar represents the percentage of branches which displayed a particular type of gradient (+, 0, or -). The reinnervated muscles were also subdivided into early (8 to 10 days postcrush) and late (12 to 30 days postcrush) stages of reinnervation. The numbers in parentheses are the total number of nerve terminal branches sampled.

when one would most likely expect positive gradients, none were observed in the 11 nerve terminals sampled. When the results from all reinnervated muscles were pooled, the bar graph was very similar to that for normals. Thus, there was no detectable spatial variation in mean mepp size to indicate the existence of a developmental gradient along the growing nerve process.

Mepp frequency. Focal mepp frequency was examined next for the existence of gradients. After ascertaining that field sizes were similar and that no obvious damage had been done by the extracellular electrode (see "Materials and Methods"), nerve terminals were given an appropriate gradient designation based on focally recorded mepp frequencies. Figure 9 is a bar graph showing the relative frequency of each type of gradient for normal and early (8 to 10 days postcrush), late (12 to 30 days postcrush), and combined (8 to 30 days postcrush) reinnervated muscles. There was an equal probability that any one type of gradient in mepp frequency existed for early reinnervated muscle fibers. The incidence in the number of positive gradients was approximately the same for all experimental times in comparison to control levels. The higher percentage of negative gradients at early times after nerve crush might be due to normal variability; even in control muscles, over half of the nerve terminals displayed differences in mepp frequency along their lengths. Clearly, regenerating nerve terminals do not appear to develop in a differential manner with regard to localized mepp frequency.

Evoked responses. Since the results on mepp frequency revealed no developmental gradients, nerve-evoked release was also studied to determine if the depolarization-secretion mechanism might mature in a different manner from spontaneous release. Because the morphological reinnervation of cp muscle is essentially complete by day 12, it was decided to concentrate on the early stage (6 to 12 days postcrush) of reinnervation.

After recording epps for quantal content calculation in low calcium/high magnesium frog Ringer's solution, one

or two extracellular electrodes were positioned along the nerve terminal. In most cases, intracellular epps were simultaneously recorded along with extracellular events to monitor possible damage (see "Materials and Methods"). Figure 7B shows representative examples of oscilloscope traces for intracellular (*top trace*) and extracellular (*bottom traces*) recordings. When there was no demonstrable electrode-induced nerve injury and the average extracellular amplitudes were of similar size (see "Materials and Methods"), terminal branches were classified into the appropriate gradient category for focally recorded evoked release. Figure 10 shows the results from these experiments. The reinnervated endplates showed a substantially higher frequency of positive gradients compared to negative gradients. However, since the control group had an even greater percentage of positively oriented gradients with no terminal branches displaying negative gradients, it must be concluded that the exper-

Figure 9. Percentage of type of gradient for mepp frequency in normal and reinnervated muscles. Each bar represents the percentage of branches which displayed a particular type of gradient (+, 0, or -). The reinnervated muscles were also subdivided into early (8 to 10 days postcrush) and late (12 to 30 days postcrush) stages of reinnervation. The numbers in parentheses are the total number of nerve terminal branches sampled.

Figure 10. Percentage of type of gradient (+, 0, or -) for the probability of evoked release. Both control and reinnervated (6 to 12 days postcrush) muscles are shown. The numbers in parentheses are the numbers of nerve terminal branches sampled.

imental muscles show no tendency for positive gradients with respect to evoked release.

While the above results give no indication of gradients being involved in the functional recovery of transmitter release, there were some results suggesting that, in a few cases, the distal length of a regenerating nerve terminal process does not function as well as the proximal portion. Out of 25 terminal branches in experimental cps examined for evoked release along their lengths, five (20%) had distal portions with no detectable evoked release in response to at least 100 nerve stimuli. While positioning of the focal recording electrode may be responsible for this result, nerve terminals from normal cps always showed release along their entire lengths ($n = 18$). As an example, Figure 11A shows a camera lucida drawing of a reinnervated endplate 6 days after nerve crush. The terminal arborization had one branch (*top right*) with no nerve-evoked extracellular events along the distal half (indicated with *arrowheads*).

In addition to occasional cases in which release was not detected at distal points, release was not seen along the few overgrowing processes which were sampled. Possibly this was due to insignificant numbers of subneural acetylcholine receptors (AChRs). Figure 11A does show, however, that release can occur within 10 μ m from the point of overgrowth. In seven out of eight cases, evoked transmitter release was observed at the point at which the overgrowth began. Since origin of the evoked responses may lie to either side of the recording site, these results do not yield information as to the ability of the distal tip of a growing nerve to release transmitter.

The question of whether there is release at a growing tip cannot be easily answered by recording from overgrowing nerve processes. However, the problem can be approached by recording from growing tips of nerve when they are still within the postsynaptic gutter. With the optics available, it was very difficult to determine where the nerve process ended within the depression of the postsynaptic membrane. Only after comparisons of the Polaroid micrograph of the living preparation with the stained endplate was it possible to determine the position

Figure 11. Camera lucida drawings of endplates from 6 day reinnervated muscles. Stippled areas are myelinated preterminal axons. Solid arrowheads indicate sites of recorded responses to nerve stimulation, while at the open arrowheads, no responses were seen. A, Example of lack of release at distal points (far right nerve branch). The last segment of this nerve branch was not checked for release because the branch could not be visualized beyond the second open arrowhead. B, At the arrow where there is an empty gutter ahead of the growing nerve process, there was also evoked release. Bar = 10 μ m.

of the extracellular pipette relative to the end of the nerve process. On four occasions, the extracellular electrode was placed at the end of a growing nerve process. Since partially filled gutters of postsynaptic membrane were rarely seen at later stages of reinnervation, it was assumed that these nerve processes were still growing and likely would have reinnervated the entire gutter within a short period of time. All four of the growing tips sampled demonstrated the ability to release transmitter. Figure 11B is a drawing of one of these neuromuscular junctions. From the nerve tip, marked with the *arrow-head*, it was possible to record focal responses to nerve stimulation. It appears, therefore, that even the tip of a regenerating nerve can release transmitter.

Discussion

The purpose of this study was to examine functional recovery following nerve crush. The first step entailed outlining morphological reinnervation and then comparing this aspect of recovery with functional maturation. Using the NBT/AChE technique (Letinsky and DeCino, 1980), it was shown that regenerating axons returned to the original endplate sites rapidly after an initial degeneration period lasting approximately 4 days after nerve crush. Ninety-five percent of the subsynaptic membrane was covered by nerve within 12 days. This time course of reinnervation is similar to that observed by Letinsky et al. (1976) and Ding (1980) on the same muscle.

The maturation of functional parameters proceeded along a much slower time course. Normalized mepp frequency never returned to control levels during the 30 days examined. In other reports on reinnervated muscle, mepp frequency was also low initially (Miledi, 1960; Bennett et al., 1973a; McArdle and Albuquerque, 1973; Dennis and Miledi, 1974b; Ding, 1980). Developing neuromuscular systems initially have low mepp frequencies (Diamond and Miledi, 1962; Letinsky, 1974), but this is probably due to the small size of the endplate region (Letinsky and Morrison-Graham, 1980) in contrast to the system in the present study. By correlating structural and functional recovery, it is possible to conclude that the maturation of spontaneous release does not depend simply upon the presence of a normal length of nerve terminal. Both Ding (1980; staining nerve and AChE in frog cp) and McArdle and Sansone (1977; staining for AChE in mammalian muscle) found similar discrepancies in morphological and mepp frequency recovery following nerve crush. Quantal size (average mepp amplitude $\div R_{in}$), on the other hand, remained fairly constant during reinnervation with most amplitude histograms being normally distributed.

As with mepp frequency, the return of quantal content to normal levels was shown to lag behind morphological recovery. Functional nervous control of the muscle resumed rapidly with suprathreshold epps present in many muscle fibers as early as 6 days after crush. Complete recovery of quantal content to control levels, however, required about 22 days. Except for Ding (1980), other investigators have not documented functional (i.e., nerve-evoked transmission) and structural recovery during reinnervation, but many have found that epp amplitude is initially low when reinnervation begins and subse-

quently increases (Bennett et al., 1973a, b; Bennett and Florin, 1974; Dennis and Miledi, 1974b). Ding's (1980) work on quantal content recovery in this muscle comes from magnesium-blocked nerve terminals; altered sensitivity to divalent cations may explain his observation of quantal contents at 30 days of about half (although not statistically different) the control levels.

Ding (1980), in his ultrastructural study on regenerating frog nerve terminals, detected differences in some morphological parameters which might partially explain the discrepancy between functional and structural recovery. Of particular interest was the finding that the number of synaptic vesicles was substantially lower than normal at 12 days postcrush. By 30 days, this parameter had returned to control levels. This ultrastructural data is consistent with the recovery seen in quantal content in the present study. It does not account, however, for the maintained depression in mepp frequency.

The results in the present study are markedly different from those of Dennis and Miledi (1971, 1974a, b). For example, they saw a large percentage of muscle fibers with skewed mepp histograms early in reinnervation, due to greater than normal numbers of small mepps; only a few such cases were observed in the present report. In addition, nontransmitting endplates and endplates with subthreshold epps were rarely seen in the current study. These discrepancies may arise because of the protracted time course of reinnervation in *Rana temporaria* compared to *Rana pipiens*. Dennis and Miledi (1974a) commented that the most medial fibers of the cp do not become reinnervated until 17 to 22 days postcrush. In contrast, the medial fibers in the cp of *Rana pipiens* are reinnervated by 8 to 10 days postcrush. Such a species difference in frogs with respect to nerve section has been reported elsewhere (Verma and Pecot-Dechavassine, 1977). The rapid time course of recovery in *Rana pipiens* may shorten the duration of recovery and hence, skewed amplitude histograms, nontransmitting endplates, and subthreshold epps are probably more difficult to detect.

Experiments examining focal transmitter release were undertaken to ascertain the mechanism of functional recovery at the reinnervated amphibian neuromuscular junction since morphological reinnervation occurred much faster than functional recovery. The results of classifying nerve terminal branches into categories according to the presence or absence of gradients for the frequency of both spontaneous and evoked transmitter release showed no indication that the proximal portion of the regenerated nerve branch was more likely to release transmitter than the distal portion. As with mepp amplitude, though, it was clear that there was a large fraction of sampled endplates in both reinnervated and normal muscles showing non-uniformity of spontaneous and evoked release. These results are consistent with the recent investigation of Bennett and Lavidis (1979) on the unoperated iliofibularis muscle of the toad which showed considerable variability in the probability of evoked release at different localized areas of nerve terminal. On occasion, they recorded extracellularly from areas which contributed up to 50% of the intracellularly determined quantal content. If the data on focally recorded evoked responses from normal muscles in the present study are

analyzed in the same manner as for the Bennett and Lavidis (1979) paper, my results show a much lower contribution from focal sites to the total quantal output of the nerve process. Using similar calcium/magnesium concentrations (0.4 mM/2 mM in the present situation versus 0.35 mM/1.2 mM in the experiments of Bennett and Lavidis), they found a large fraction (11 sites/28 different nerve terminals) of focal recording sites which contributed 20 to 50% of the total terminal output (Fig. 4, Bennett and Lavidis, 1979). The control endplates in the current investigation demonstrated no release sites ($n = 41$) which contributed more than 11% (P. DeCino, unpublished observation). The disparity may be due to species or muscle differences resulting from varying calcium sensitivities or to differences in techniques.

While there were no proximal-distal gradients for evoked release, a few regenerating terminals lacked the ability to release transmitter along their distal portions. This observation may be due to action potential failure with electrotonic conduction which allowed only the proximal portion to reach the threshold level for transmitter release. The nerve action potential was not recorded in the present experiments but Dennis and Miledi (1974a) have shown action potential conduction block to be responsible for nontransmitting endplates. A differential sensitivity to calcium along a nerve terminal which prevents release at distal points is not a likely mechanism since there were no positive gradients.

The results which show release at or very near ($<10 \mu\text{m}$) growing tips indicate that presumably new portions of nerve terminal membrane are sufficiently mature to permit depolarization-secretion coupling. In culture systems where it is easier to monitor the onset of synaptic activity, Kidokoro (1980) has observed mepps within 2 hr of the addition of spinal cord explants to cultured rat primary myotubes. Furthermore, Cohen (1980), using chick cultures, recorded epps 30 min after nerve-muscle contact.

The lack of convincing evidence for positive gradients for the frequency of transmitter release suggests that the entire length of a regenerating nerve process matures at nearly the same rate. Although individual nerve processes cannot be observed growing into an endplate region without using an organ culture system, the rapidity of morphological reinnervation indicates that individual gutters, corresponding to particular nerve branches, are likely filled within 1 to 2 days once reinnervation of that gutter commences. This small difference in age of the proximal versus distal portion of nerve terminal may not permit detection of gradients when one considers the relatively slow time course of the return to normal levels of mepp frequency and quantal content.

The experiments presented here have demonstrated temporal differences in the morphological and physiological time course of reinnervation in frog muscle. In addition, the results have shown that no obvious gradients for the probability of evoked release appear in the course of a nerve process reinnervating an established postsynaptic region. There were instances, though, in which the distal portion of a nerve branch showed no sign of focal responses. Of interest, also, is the result showing that the

tip of a growing nerve process could release neurotransmitter upon nerve stimulation.

References

- Anderson, M. J., Y. Kidokoro, and R. Gruener (1979) Correlation between acetylcholine localization and spontaneous synaptic potentials in cultures of nerve and muscle. *Brain Res.* 166: 185-190.
- Bennett, M. R., and T. Florin (1974) A statistical analysis of the release of acetylcholine at newly formed synapses in striated muscle. *J. Physiol. (Lond.)* 238: 93-107.
- Bennett, M. R., and N. A. Lavidis (1979) The effect of calcium ions on the secretion of quanta evoked by an impulse at nerve terminal release sites. *J. Gen. Physiol.* 74: 429-456.
- Bennett, M. R., and A. G. Pettigrew (1975) The formation of synapses in amphibian striated muscle during development. *J. Physiol. (Lond.)* 252: 203-239.
- Bennett, M. R., A. G. Pettigrew, and R. S. Taylor (1973a) The formation of synapses in reinnervated and cross-reinnervated adult avian muscle. *J. Physiol. (Lond.)* 230: 331-357.
- Bennett, M. R., E. M. McLachlan, and R. S. Taylor (1973b) The formation of synapses in reinnervated mammalian striated muscle. *J. Physiol. (Lond.)* 233: 481-500.
- Cohen, S. A. (1980) Early nerve-muscle synapses *in vitro* release transmitter over postsynaptic membrane having low acetylcholine sensitivity. *Proc. Natl. Acad. Sci. U. S. A.* 77: 644-648.
- Dennis, M. J., and R. Miledi (1971) Lack of correspondence between the amplitudes of spontaneous potentials and unit potentials evoked by nerve impulses at regenerating neuromuscular junctions. *Nature* 232: 126-128.
- Dennis, M. J., and R. Miledi (1974a) Non-transmitting neuromuscular junctions during an early stage of end-plate reinnervation. *J. Physiol. (Lond.)* 239: 553-570.
- Dennis, M. J., and R. Miledi (1974b) Characteristics of transmitter release at regenerating frog neuromuscular junctions. *J. Physiol. (Lond.)* 239: 571-594.
- Diamond, J., and R. Miledi (1962) A study of foetal and newborn rat muscle fibres. *J. Physiol. (Lond.)* 162: 393-408.
- Ding, R. (1980) Physiology and morphology of synapses formed *de novo* and at old endplates by regenerating frog nerves. Doctoral thesis, U.C.L.A.
- Duncan, C. J., and S. J. Publicover (1979) Inhibitory effects of cholinergic agents on the release of transmitter at the frog neuromuscular junction. *J. Physiol. (Lond.)* 294: 91-103.
- Furshpan, E. J. (1956) The effects of osmotic pressure changes on the spontaneous activity at motor nerve endings. *J. Physiol. (Lond.)* 134: 689-697.
- Grinnell, A. D., and A. A. Herrera (1980) Physiological regulation of synaptic effectiveness at frog neuromuscular junctions. *J. Physiol. (Lond.)* 307: 301-318.
- Hutter, O. F., and W. Trautwein (1956) Neuromuscular facilitation by stretch of motor nerve endings. *J. Physiol. (Lond.)* 133: 610-625.
- Karnovsky, M. J., and L. Roots (1964) A direct-coloring thiocholine method for cholinesterases. *J. Histochem. Cytochem.* 12: 219-221.
- Kidokoro, Y. (1980) Developmental changes of spontaneous synaptic potential properties in the rat neuromuscular contact formed in culture. *Dev. Biol.* 78: 231-241.
- Kriebel, M. E., and C. E. Gross (1974) Multimodal distribution of frog miniature endplate potentials in adult, denervated and tadpole leg muscle. *J. Gen. Physiol.* 64: 85-103.
- Kuno, M., S. A. Turkanis, and J. N. Weakly (1971) Correlation between nerve terminal size and transmitter release at the neuromuscular junction of the frog. *J. Physiol. (Lond.)* 213: 545-556.

- Letinsky, M. S. (1974) The development of nerve-muscle junctions in *Rana catesbeiana* tadpoles. *Dev. Biol.* 40: 129-153.
- Letinsky, M. S., and P. A. DeCino (1980) Histological staining of pre- and postsynaptic components of amphibian neuromuscular junctions. *J. Neurocytol.* 9: 305-320.
- Letinsky, M. S., and K. Morrison-Graham (1980) Structure of developing frog neuromuscular junctions. *J. Neurocytol.* 9: 321-342.
- Letinsky, M. S., K. H. Fischbeck, and U. J. McMahan (1976) Precision of reinnervation of original postsynaptic sites in frog muscle after a nerve crush. *J. Neurocytol.* 5: 691-718.
- Martin, A. R. (1960) Quantal nature of synaptic transmission. *Physiol. Rev.* 46: 51-66.
- McArdle, J. J., and E. X. Albuquerque (1973) A study of the reinnervation of fast and slow mammalian muscles. *J. Gen. Physiol.* 61: 1-23.
- McArdle, J. J., and F. M. Sansone (1977) Reinnervation of fast and slow twitch muscle following nerve crush at birth. *J. Physiol. (Lond.)* 271: 567-586.
- McMahan, U. J., and S. W. Kuffler (1971) Visual identification of synaptic boutons on living ganglion cells and of varicosities in postganglionic axons in the heart of the frog. *Proc. R. Soc. Lond. (Biol.)* 177: 485-508.
- Miledi, R. (1966) Properties of regenerating neuromuscular synapses in the frog. *J. Physiol. (Lond.)* 154: 190-215.
- Rotshenker, S. (1979) Synapse formation in intact innervated cutaneous pectoris muscles of the frog following denervation of the opposite muscle. *J. Physiol. (Lond.)* 292: 535-547.
- Verma, V., and M. Pecot-Dechavassine (1977) A comparative study of physiological and structural changes at the myoneural junction in two species of frog after transection of the motor nerve. *Cell Tissue Res.* 185: 451-464.
- Wernig, A. (1976) Localization of active sites in the neuromuscular junction of the frog. *Brain Res.* 118: 63-72.