Regeneration of the Active Zone at the Frog Neuromuscular Junction

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ABSTRACT The active zone is a unique specialization of the presynaptic membrane and is believed to be the site of transmitter release. The formation of the active zone and the relationship of this process to transmitter release were studied at reinnervated neuromuscular junctions in the frog. At different times after a nerve crush, the cutaneous pectoris muscles were examined with intracellular recording and freeze-fracture electron microscopy.

The P face of a normal active zone typically consists of two double rows of particles lined up in a continuous segment located opposite a junctional fold. In the initial stage of reinnervation, clusters of large intramembrane particles surrounding membrane elevations appeared on the P face of nerve terminals. Like normal active zones, these clusters were aligned with junctional folds. Vesicle openings, which indicate transmitter release, were seen at these primitive active zones, even though intramembrane particles were not yet organized into the normal pattern of two double rows. The length of active zones at this stage was only ~15% of normal.

During the secondary stage, every junction was reinnervated and most active zones had begun to organize into the normal pattern with normal orientation. Unlike normal, there were often two or more discontinuous short segments of active zone aligned with the same junctional fold. The total length of active zone per junctional fold increased to one-third of normal, mainly because of the greater number of segments.

In the third stage, the number of active zone segments per junctional fold showed almost no change when compared with the secondary stage. However, individual segments elongated and increased the total length of all active zone segments per junctional fold to about two-thirds of the normal length.

The dynamic process culminated in the final stage, during which elongating active zones appeared to join together and the number of active zone segments per junctional fold decreased to normal. Thus, in most regions, regeneration of the active zones was complete.

These results suggest that the normal organization of two double rows is not necessary for the active zone to be functional. Furthermore, localization of regenerating active zones is related to junctional folds and/or their associated structures.

One of the key structures at the synapse is the site of transmitter release, which has been called the active zone (5). At the frog neuromuscular junction, thin-section electron microscopy has shown that the active zone has electron-dense material on the presynaptic membrane and is located over the opening of the junctional fold. In addition, synaptic vesicles tend to cluster around the active zone (1). Freeze-fracture of the frog neuromuscular junction has provided an *en face* view of the active zone and has revealed even more dramatically its unique organization and function. On the protoplasmic (P)[1] face, the active zone has a membrane ridge bordered by two double rows of large intramembrane particles and is located precisely opposite the opening of the junctional fold (9, 26). During transmitter release, synaptic vesicles fuse with the presynaptic membrane preferentially at the edges of

[1] *Abbreviations used in this paper:* EPP, endplate potential; P face, protoplasmic face; pd, post-denervation.

the active zone (3, 14). These vesicle fusions are thought to account for the quantal release of neurotransmitter (12, 13). These freeze-fracture studies indicate that the active zone plays an important role in transmitter release.

During degeneration of frog neuromuscular junctions, disruption of active zones occurs at about the same time as transmission failure (16). The nerve terminals then disintegrate and are engulfed by Schwann cells. Later, Schwann cells occupy the entire junctional gutter and develop membrane ridges opposite the junctional folds. However, the ridges on Schwann cells do not show an organization of particles similar to that found at nerve terminals (15, 16).

The purpose of the present work was to study the next step, the reformation of active zones when nerve terminals reinnervate neuromuscular junctions after a nerve crush. Heuser (10) reported that active zones at regenerating terminals are discontinuous structures composed of several short segments of active zone particles. However, the entire sequence of active zone regeneration and its relation to functional changes have not been studied. It has not been shown whether the active zone regenerates to its normal configuration and, if it does, how the active zone acquires its unique organization and precise location in relation to the junctional fold. Furthermore, it is not known at what stage of morphogenesis the active zone becomes functional. In order to answer these questions and better understand the unique nature of the active zone, reinnervation of frog neuromuscular junctions was studied by combining intracellular recording and freeze-fracture electron microscopy. A preliminary report of these results has been published (17).

MATERIALS AND METHODS

Operation: Cutaneous pectoris muscles of adult northern grass frogs (*Rana pipiens*) were used. Frogs (4–5 cm body length) were kept at room temperature and fed crickets twice a week. After the animal had been anesthetized by immersion in 0.1% tricaine methane sulfonate (MS 222; Sigma Chemical Co., St. Louis, MO), nerves to both right and left muscles were crushed with fine forceps at a distance 2–3 mm from the point of nerve attachment to the muscles. At different times (10–570 d) after a nerve crush, the muscles were dissected and bathed in frog Ringer's solution composed of 111 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES at pH 7.2.

Electrophysiology: After dissection of the muscles, conventional intracellular recordings were performed at room temperature. To monitor the functional state of reinnervation, the nerve was stimulated with a suction electrode and endplate potentials (EPPs) were recorded with a glass microelectrode while the muscle was bathed in Ringer's solution containing 2.1×10^{-6} g/cc of *d*-tubocurarine or Ringer's solution containing 4 mM of $MgCl_2$ and 0.7 mM $CaCl_2$.

Freeze-Fracture Electron Microscopy: After intracellular recording the same muscles were fixed with 3% glutaraldehyde in Ringer's solution for 1 h and then glycerinated (16). Neuromuscular regions were dissected out and placed between two specimen carriers and frozen with Freon-22. The frozen muscles were stored in liquid N_2 and then fractured by a complementary replica device in a Balzers 301 or 400D freeze-fracture apparatus (Balzers Co., Nashua, NH).

To capture the process of transmitter release, some muscles were stimulated during chemical fixation (14). The preparation was first submerged in Ringer's solution containing 10^{-5} g/cc of *d*-tubocurarine. After the muscle twitch was blocked by *d*-tubocurarine, the above solution plus 0.75% formaldehyde was introduced. Within 30 s of the time at which the preparation was first exposed to this dilute fixative, the nerve was stimulated at 10 Hz for 4 min. After this treatment muscles were placed in 3% glutaraldehyde and the normal procedures for freeze-fracture (described above) were followed.

The morphometry of active zone segments was performed with a digitizing table (Bitpad; Summagraphics Corp., Fairfield, CT) interfaced with a microcomputer (Horizon 2, North Star Computers, Inc., San Leandro, CA). In some cases, especially during regeneration, active zones were not continuous. If active zones facing the same junctional fold were separated by a gap of >50 nm, they were considered as two separate segments (27). Accordingly, the length of each of these individual segments of active zones was measured, as well as the total lengths of all segments per junctional fold. In addition, the number of segments per junctional fold was calculated.

RESULTS

Normal Active Zone

The active zone at the normal frog neuromuscular junction has been well described (see reference 10). In order to compare regenerating active zones with normal active zones, a freeze-fractured neuromuscular junction of a normal frog is shown in Fig. 1. On the P face of the nerve terminal the active zone can easily be recognized as the transverse ridge bordered on each side by double rows of large intramembrane particles with diameters of ~9–11 nm. These active zone particles are located just opposite a junctional fold and typically run perpendicular to the longitudinal axis of the terminal. On the extracellular face of the terminal the active zone is marked by a groove that is complementary to the ridge seen on the P face and has only a few large particles. In ~80% of the cases there is only one continuous segment of active zone opposite each junctional fold. In the remainder there are two or more discontinuous segments. On the average there are 1.26 segments of active zone per junctional fold. The average length of each individual active zone segment is 976 nm and the average total length of all active zone segments aligned to one junctional fold is 1.245 μm (in seven muscles) (Table I).

Occasionally, when a piece of nerve terminal was cross-fractured, synaptic vesicles (arrowhead) were seen, as shown in Fig. 1 *a*. A dimple (arrow), which may represent an opening of a synaptic vesicle, was occasionally found at an active zone. When the nerve was stimulated in dilute formaldehyde (see Materials and Methods), more vesicle openings were found at both sides of the active zone (Fig. 1 *b*). Dimples were never observed at the elevated ridge between the two double rows of active zone particles.

Following degeneration of nerve terminals, postjunctional folds remain unchanged for a period of time and can serve as a marker for the endplate site during the study of regeneration (16, 20, 29). Regeneration is a dynamic process. Within a given muscle, not all active zones regenerate synchronously. In order to facilitate the understanding of this continuous process of regeneration, the results are organized into the following four stages. The purpose of these stages is to describe the general trend of how active zones regenerate from a cluster of particles to the normal pattern.

Initial Stage of Regeneration

The first sign of functional reinnervation occurred at about 13 d after a nerve crush. At this time, some muscle fibers began to show EPPs upon nerve stimulation. The percentage of neuromuscular junctions showing evoked EPPs increased with time and reached 100% at about 19 d post-denervation (pd).

During this initial period of reinnervation, endplates presented a variety of different appearances in freeze-fracture replicas. Some endplates were still denervated and occupied by Schwann cells, as shown previously (15, 16). Some junctions were beginning to show additional structures, which were probably regenerating nerve terminals. Even though it was difficult to positively identify these structures as regenerating nerve terminals, most of them already displayed distinct

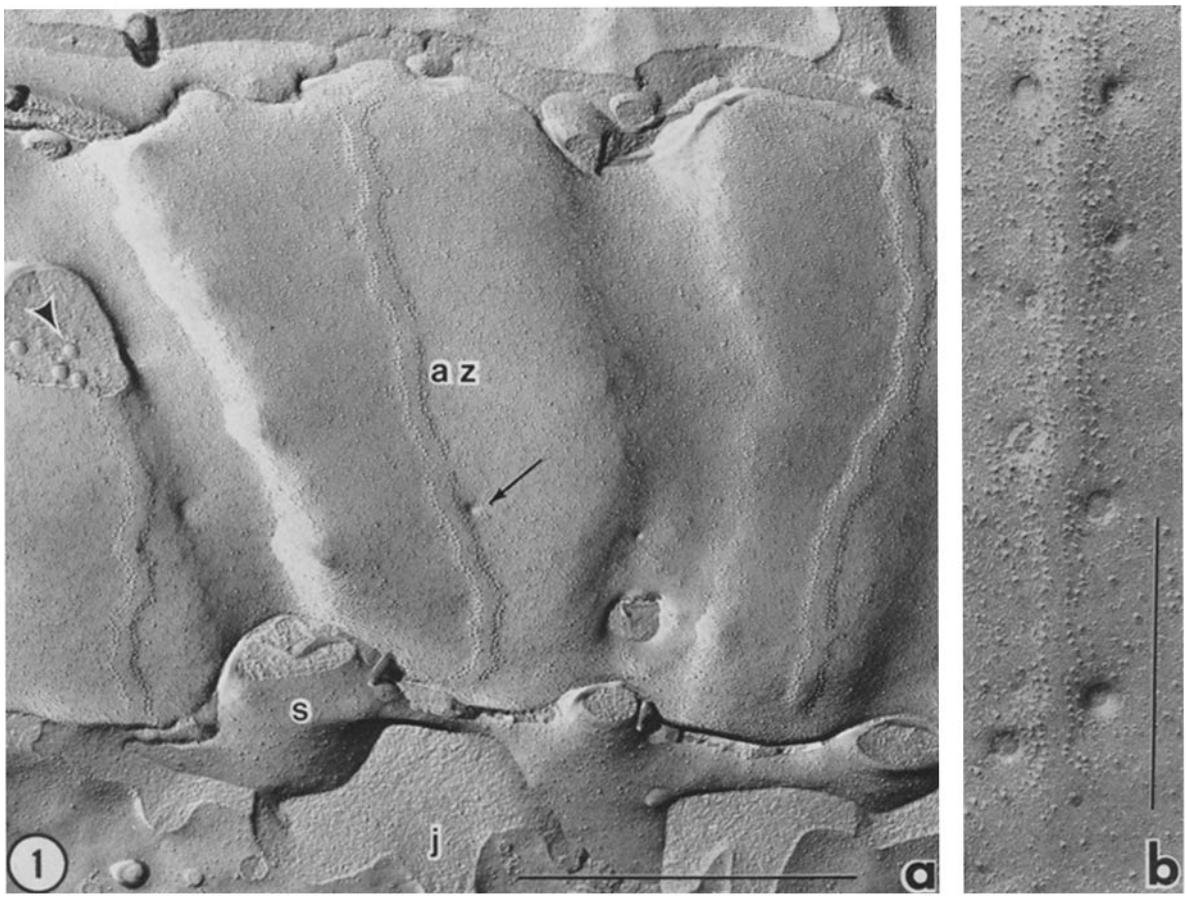


FIGURE 1 Nerve terminals (P face) in normal neuromuscular junctions fixed at rest (*a*) and during stimulation (*b*). In *a*, active zones (*az*), with two double rows of large particles, are located opposite junctional folds (*j*). A portion of this nerve terminal was cross-fractured and revealed synaptic vesicles (arrowhead). A dimple (arrow) is seen next to an active zone. Schwann cell processes (*s*) separate neighboring active zones. In *b*, numerous dimples are found at both sides of the active zone. This nerve was stimulated at 10 Hz for 4 min in 0.75% formaldehyde. In this and the following electron micrographs, platinum deposits are black and shadows are white. (*a*) Bar, 1 μm. × 45,000. (*b*) Bar, 0.5 μm. × 78,000.

TABLE I

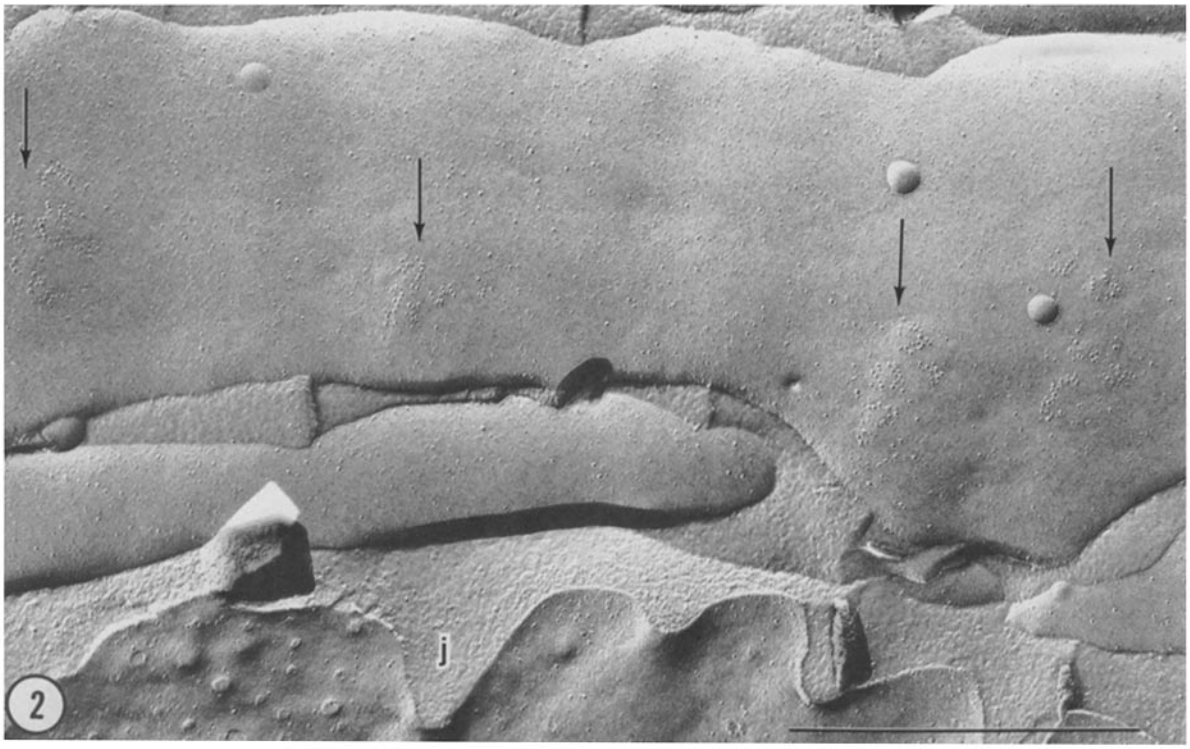
Morphometry of Active Zones in Normal and Reinnervated Neuromuscular Junctions

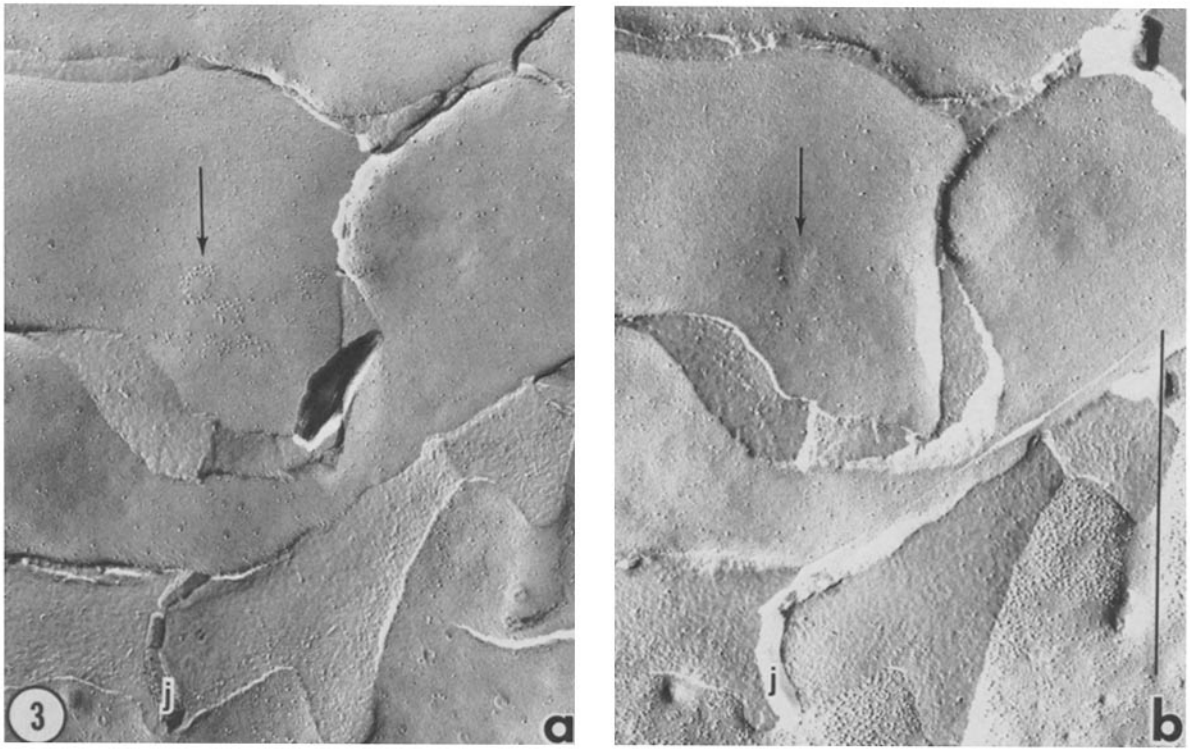
Days pd	Stage	Number of endplates	Number of active zones per junctional fold		Lengths of individual active zones		Length of total active zones per junctional fold	
			mean ± SD		*mean (nm) ± SD*		*mean (nm) ± SD*	
—	Normal	12	1.26 ± 0.51	193*	976 ± 530	244*	1,245 ± 659	193*
15	I	6	1.03 ± 0.19	29	188 ± 89	30	194 ± 89	29
18	I	3	1.17 ± 0.39	12	160 ± 61	14	186 ± 65	12
22	II	1	1.33 ± 1.58	3	161 ± 44	4	218 ± 59	3
28	II	12	1.86 ± 0.97	164	228 ± 146	305	421 ± 238	164
78	III	10	1.89 ± 0.89	123	436 ± 317	232	828 ± 428	123
480	IV	6	1.06 ± 0.25	62	886 ± 390	66	943 ± 380	62
576	IV	4	1.56 ± 0.84	54	789 ± 482	84	1,225 ± 603	54

* Values in these columns indicate the number of measurements.

clusters of large intramembrane particles (arrows in Fig. 2) on the P face. Similar to normal active zone particles, these particles had diameters of ~9–11 nm and were located conspicuously just opposite junctional folds; however, these particles were not organized into the "railroad track" pattern, as seen at the normal active zones. Since Schwann cells at denervated junctions do not have clusters of intramembrane particles (16), structures with assemblies of particles observed at this stage probably belong to regenerating nerve terminals and these assemblies may represent the initial forms of active zones.

In addition to clusters of large intramembrane particles, membrane ridges, normally associated with active zones, are also found at regenerating nerve terminals. These membrane elevations became more apparent by examining complementary replicas as shown in Fig. 3. On the P face there is a cluster





FIGURES 2 and 3 Fig. 2: Nerve terminal (P face) during the initial stage of active zone regeneration, 13 d pd. Clusters of large intramembrane particles (arrows) appear aligned with junctional folds (*j*). In this muscle, 30% of the junctions showed EPPs. Bar, 1 μm. × 45,000. Fig. 3: Complementary replicas of regenerating active zones, 13 d pd. (*a*) P face of nerve terminal. A cluster of active zone particles (arrow) with an elevated membrane is aligned with a junctional fold. (*b*) E face of the same terminal. A complementary membrane depression (arrow) is found at the same location as the membrane elevation in *a*. Bar, 1 μm. × 45,000.

of particles surrounding a ridge (arrow in Fig. 3*a*). Because of the presence of the particles, this ridge was not easily noticeable. However, on the extracellular face of the same nerve terminal, where only a few particles are present, a depression (arrow in Fig. 3*b*) that is complementary to the ridge on the P face can be readily recognized. As in normal active zones, membrane ridges or depressions have not been seen to extend beyond the clusters of active zone particles. These findings

indicate that the membrane ridge appears quite early and is closely associated with the active zone particles during regeneration.

During this early stage of regeneration, most active zone assemblies were very short. At 13 d pd, it was difficult to measure active zones accurately because of the difficulty in defining the boundaries of clusters of particles. After 15 d pd, more active zone clusters were in an elongated form with normal orientation and thus morphometric measurements were possible. At most regions (95%) there was only one segment of primitive active zone opposite to one junctional fold (Fig. 4*a*, histogram). The average number of active zone segments per junctional fold was 1.03 at 15 d pd and 1.17 at 18 d pd (Table I and Fig. 4*a*). The average length of individual segments was 188 nm at 15 d pd and 160 nm at 18 d pd (Table I), or only ~18% of normal (Fig. 4*b*). The total length of all segments of active zone lining up along the same junctional fold was 194 and 186 nm at 15 and 18 d, respectively (Table I), or ~15% of normal (Fig. 4*c*).

Although active zones at this initial stage of regeneration were short and lacked normal organization, they were already in the normal locations relative to junctional folds. The average distance between neighboring junctional folds was 916 ± 294 (SD) nm (n = 53) in reinnervated junctions, which was not significantly different from normal junctions: 917 ± 208 (SD) nm (n = 33). However, 80% of the regenerating active zones at this stage were already located specifically within 50 nm of nearby junctional folds (Fig. 5*b*). This distance was measured from the center of an active zone to a straight line that was perpendicular to the long axis of the terminal and intersected the center of the nearby junctional fold. This straight line could not always reflect the true position of the underlying junctional fold because it was not always exactly perpendicular to the long axis of the terminal. Nevertheless, using the same criterion, it was also found in normal muscles that 90% of active zones were seen within 50 nm of junctional folds. This point was illustrated by histograms (Fig. 5) for normal and regenerating junctions, which were found to not be significantly different (χ^2 test, $P > 0.5$). In addition, the average distance between active zones and junctional folds in both normal and reinnervated junctions was <50 nm. If active zones were located randomly, one would expect the average distance between active zones and junctional folds to be ~230 nm. Therefore, the result suggests

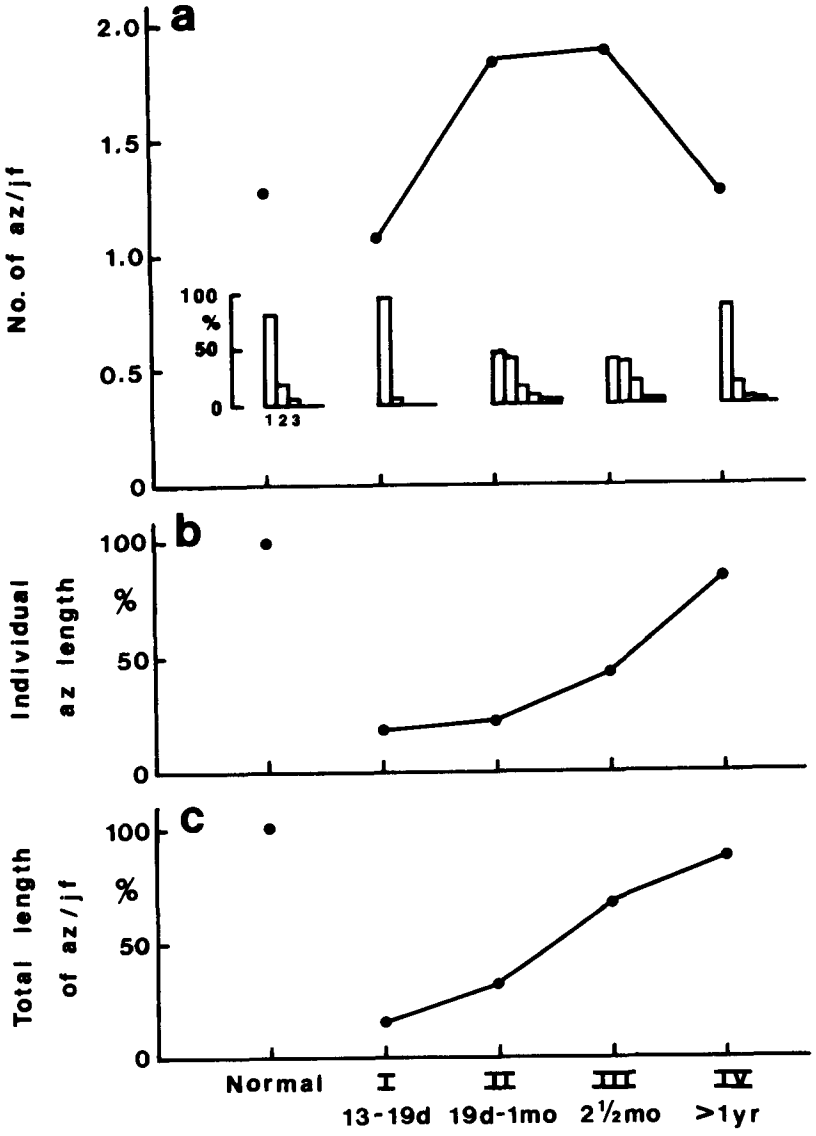


FIGURE 4 Dynamic changes in the morphometry of active zones during regeneration. The x-axis denotes normal and four stages of regeneration. In *a* the y-axis measures the number of active zone segments (*az*) per junctional fold (*jf*); in *b*, the normalized length of individual segments of active zones; and in *c*, the normalized length of all segments that are aligned with the same junctional folds. Each point represents an average of all data from the same stage. In addition, in *a*, histograms illustrate the number of active zones per junctional fold at each stage. In the initial regeneration stage (I) most active zones are short and most regions have only one active zone facing the same junctional fold. During the secondary stage (II) individual segments of active zone elongate slightly, but the number of active zone segments increases almost twofold. As shown in the histogram, more than half of the junctional folds have two or more active zone segments. As regeneration advances to stage III, there is little change in number of active zone segments, but individual segments double in length. As a result there is a further increase in the total length of active zones per junctional fold. Finally, at stage IV these elongating segments seem to join and form a single active zone at most regions.

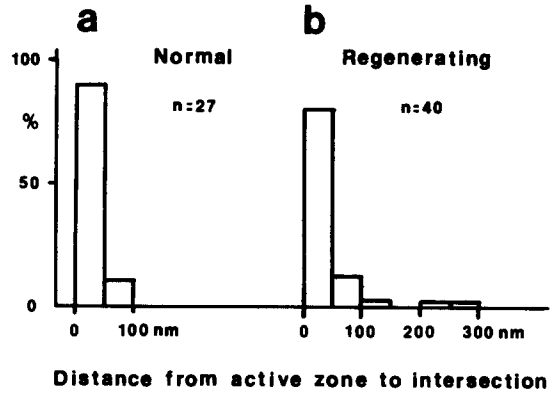


FIGURE 5 Histograms of distance from the center of active zones to the nearest intersection of a junctional fold in (*a*) normal junctions and (*b*) junctions at the initial stage of regeneration (13–19 d pd). Most normal and regenerating active zones are located within 50 nm from the intersection of a junctional fold. There is not a significant difference between the two histograms. (χ^2 test, $P > 0.5$.)

that even at the early stage of regeneration, clusters of active zone particles appear at specific regions just opposite junctional folds.

At this initial stage of reinnervation, evoked EPPs were recorded in some endplates; hence, it was conceivable that the primitive active zones were able to release neurotransmitter and produced EPPs. To study more directly whether or not these primitive active zones were functional, nerves were stimulated in the presence of dilute formaldehyde, a procedure that captured the process of exocytosis of synaptic vesicles (14). One example of this treatment is shown in Fig. 6. In Fig. 6*a* (low magnification) it is again clear that active zone clusters (arrows) are located opposite junctional folds. In addition, dimples that represent openings of synaptic vesicles (12–14) were observed at these specific regions of the presynaptic membrane. In Fig. 6*b* (higher magnification) dimples (arrowheads) were found at the primitive active zones even though these active zones were short and had only clusters of particles rather than the normal pattern of two double rows. Thus, it appears that clusters of active zone particles are already functional at this early stage of regeneration and the normal pattern of organization is not required for transmitter release.

The low magnification view of this junction (Fig. 6*a*) also illustrates the dynamic aspects of reinnervation. Normally, the nerve terminal occupied most of the area of the junctional gutter. However, it was often found that in a reinnervated junction a large area of the gutter was still occupied by the Schwann cell, and only a small portion of the gutter was covered by the nerve terminal. As the reinnervation advanced, Schwann cells retracted further and nerve terminals came to reoccupy a larger area of the junctional gutter.

Secondary Stage of Regeneration

After 19 d pd, nerve stimulation produced EPPs for every muscle fiber recorded. Also, freeze-fracture of these muscles showed that every junctional gutter was occupied by a nerve terminal. At this stage, as in the normal endplate, the regenerating nerve ending covered most of the junctional region and only a few Schwann cell processes were seen between the terminal and the muscle fiber (Fig. 7).

At this time, even though every junction was functional, close examination of freeze-fractured nerve terminals revealed that not all of the active zones had completely regenerated. Some still had only clusters of particles similar to those at the previous stage, while others within the same terminal had particles lined up in one single row or one double row with a normal orientation parallel to the junctional fold (Fig. 7, arrows). Most active zones, however, displayed the characteristic organization of two double rows of particles (Fig. 7, arrowheads). Between these two double rows was a membrane ridge devoid of large particles, similar to the normal active zone. These observations indicate that the regeneration of active zones is not synchronized within a given terminal. Although the fractured membranes represent only a portion of the nerve terminal (~10–15%), there is no apparent gradient in the fractured terminal which might suggest that regeneration of active zones starts at one end and gradually proceeds to the other end of a nerve terminal.

Regardless of their organization, most regenerating active zones at the secondary stage were still much shorter than normal, but longer than those of the initial stage. The average length of individual segments increased slightly to 228 nm at 28 d pd (Table I), or 23% of normal (Fig. 4*b*). However, the total length of all active zone segments per junctional fold increased to more than twice that of the previous stage, 421 nm at 28 d pd (Table I), or 34% of normal (Fig. 4*c*). This larger increase in total length of active zones was mainly due to an increase in the number of discontinuous active zone segments opposite the same junctional fold, which was one of the prominent characteristics at this stage. The average number of segments per junctional fold increased from 1.07 at the initial stage to 1.85 at the secondary stage, which is significantly larger than normal 1.26 (t test, $P < 0.001$) (Fig. 4*a*). As shown in the histogram, 57% of the junctional folds had two or more active zone segments (Fig. 4*a*).

Once again dimples were observed when the nerve was stimulated in dilute formaldehyde. These dimples appeared at active zones regardless of their stage of maturation (Fig. 8). Active zones with two double rows of particles, as found in the normal state, had dimples located on both sides of the active zones (Fig. 8, arrowheads). Vesicle openings were not observed on the ridges between the two double rows or at the gaps between discontinuous active zone segments facing the same junctional fold. Some active zones in the same terminal had only clusters of particles. Their vesicle openings (Fig. 8, arrows) were less organized and were intermingled with the particles rather than lined up, as normally found on both sides of the active zone. In the case of either mature or less developed active zones, the dimples were closely associated with active zone particles and were located just opposite the junctional folds.

Elongation Stage of Regeneration

At 78 d pd, the number of active zone segments per junctional fold was 1.89 and not significantly different from the previous stage (Table I). In contrast, the length of individual pieces of active zone increased significantly (t test, $P < 0.001$) from 228 nm at 28 d pd to 436 nm at 78 d pd (Table I and Fig. 4*b*). As a result, the total length of all segments per junctional fold also increased significantly (t test, $P < 0.001$) to 828 nm, which is almost twice as long as in the secondary stage (Table I and Fig. 4*c*). However, their lengths were still significantly (t test, $P < 0.001$) shorter than normal. Individual segments had only reached 45% of their normal length (Fig.

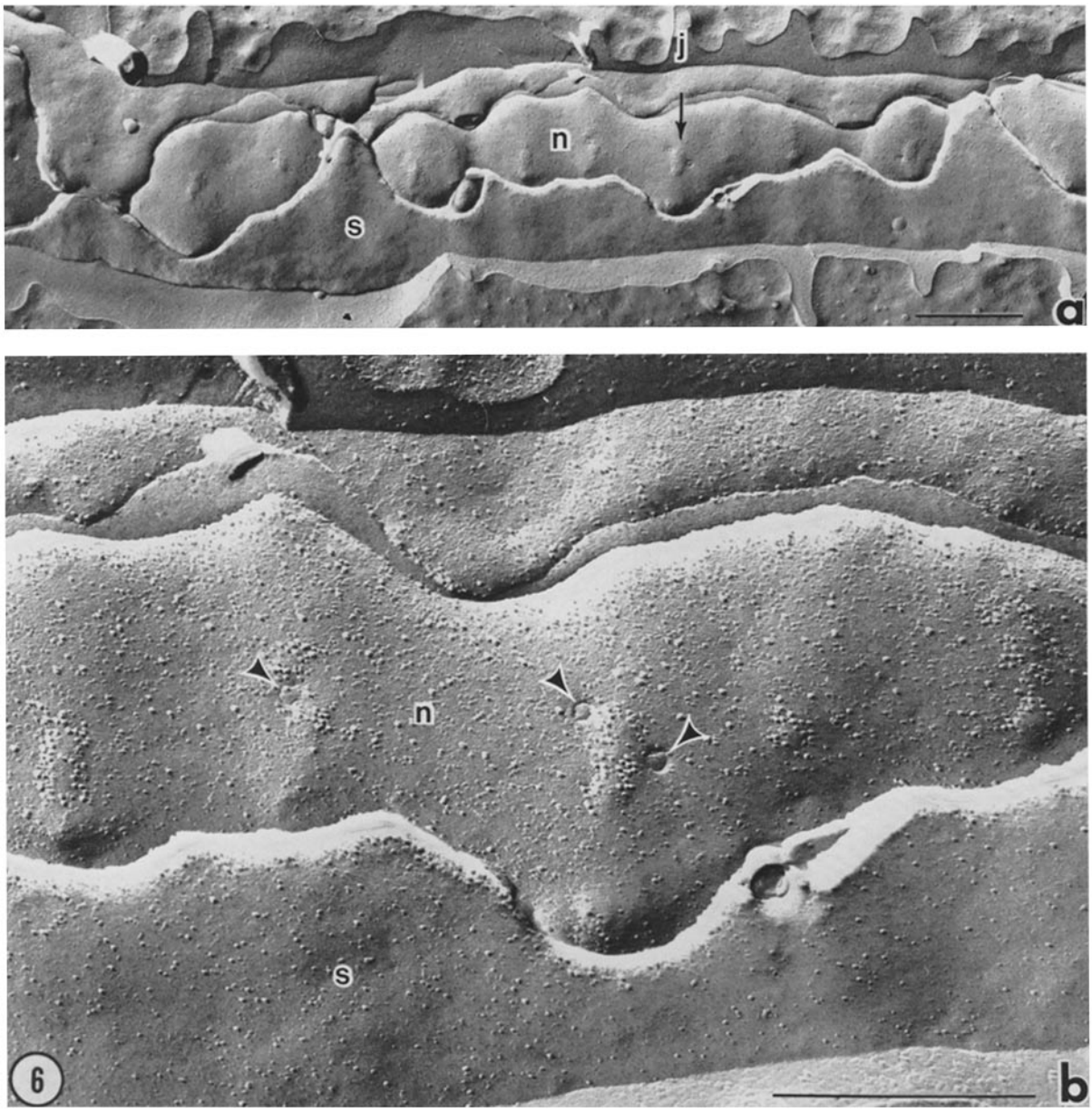
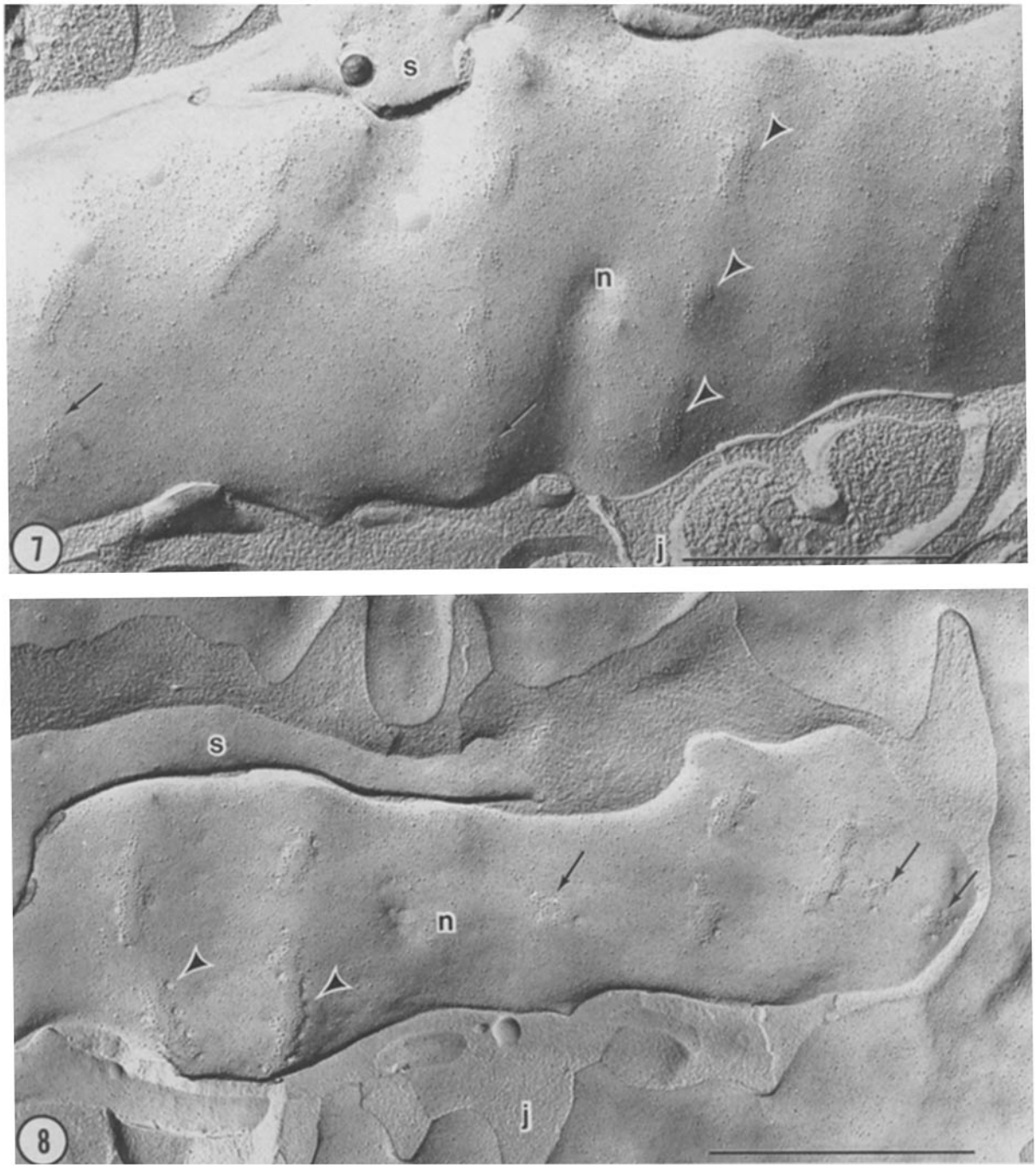


FIGURE 6 Nerve terminal at the initial stage of regeneration, 15 d pd. This nerve was stimulated at 10 Hz for 4 min in 0.75% formaldehyde. (*a*) Low magnification. Distinct clusters of active zone particles (arrow) are located on the nerve terminal (*n*) just opposite junctional folds (*j*). A large portion of the junctional gutter is still covered by Schwann cells (*s*). (*b*) High magnification of a portion of nerve terminal in *a*. Vesicle openings (arrowheads) are seen at these primitive active zones even though they have not yet organized into the normal pattern of two double rows. (*a*) Bar, 1 μm. × 16,000. (*b*) Bar, 0.5 μm. × 76,000.

4*b*). The total length of all active zone segments per junctional fold was only 67% of normal (Fig. 4*c*). Many active zones still looked immature (Fig. 9*a*), even at this length of time after the nerve crush, while other active zones (Fig. 9*b*) appeared indistinguishable from normal. Not only did these mature active zones show the typical railroad track organization of particles, they also had normal lengths and normal numbers of segments aligned to one junctional fold. When the nerve was stimulated in fixative, dimples were found bordering the active zones, as in the normal case. In addition to these vesicle openings associated with the active zone, some dimples were also seen in the regions beneath broken-away Schwann processes. These dimples may represent the endocytosis of synaptic vesicles (11, 14).

Late Stage of Regeneration

Regeneration of active zones seemed to be complete in most neuromuscular junctions examined at 480 and 570 d pd. The number of active zone segments per junctional fold decreased from 1.89 in the previous stage to an average of 1.29 (Fig. 4*a*), which was not significantly different from the normal number (*t* test, $P > 0.5$). In addition, the average length of individual active zones increased to 832 nm at this late stage or 85% of the normal length (Fig. 4*b*). Similarly, the average length of all active zone segments per junctional fold increased to 1.06 μm, or 86% of the normal length (Fig. 4*c*). Although these average values were not restored completely to normal, most active zones appeared indistinguish-



FIGURES 7 and 8 Fig. 7: Nerve terminal at the secondary stage of regeneration, 28 d pd. Some active zone particles form one single or one double row (arrows) with an orientation similar to that of normal. Most active zones, however, are beginning to organize into the normal pattern and orientation at the normal location (arrowheads). There is usually more than one active zone segment aligned with one junctional fold (*j*), which is characteristic of this stage of regeneration. In this muscle EPPs were recorded in every junction studied. Bar, 1 μm. × 41,000. Fig. 8: Nerve terminal at the secondary stage of regeneration, 28 d pd. This nerve was stimulated at 10 Hz for 4 min in 0.75% formaldehyde. Vesicle openings are found along both sides of the more mature active zones (arrowheads), and are intermingled with the less organized clusters of particles (arrows). Bar, 1 μm. × 41,000.

able from normal, especially at 576 d pd, where the total length of active zones per junctional fold was 1.225 μm, which was not significantly different from normal 1.245 μm (*t* test, $P > 0.5$) (Table I). Therefore, it seems that short active zone segments found at the earlier stages of regeneration elongate and eventually join together to form the long continuous active zone that is seen at most regions of the normal nerve terminals.

DISCUSSION

Sequence of Regeneration of Active Zones

This study illustrates the dynamic sequence of regeneration of a very important and unique structure, the active zone of the presynaptic membrane. This sequence is summarized diagrammatically in Fig. 10. At the initial stage, when EPPs begin to reappear, nerve terminals with clusters of active zone

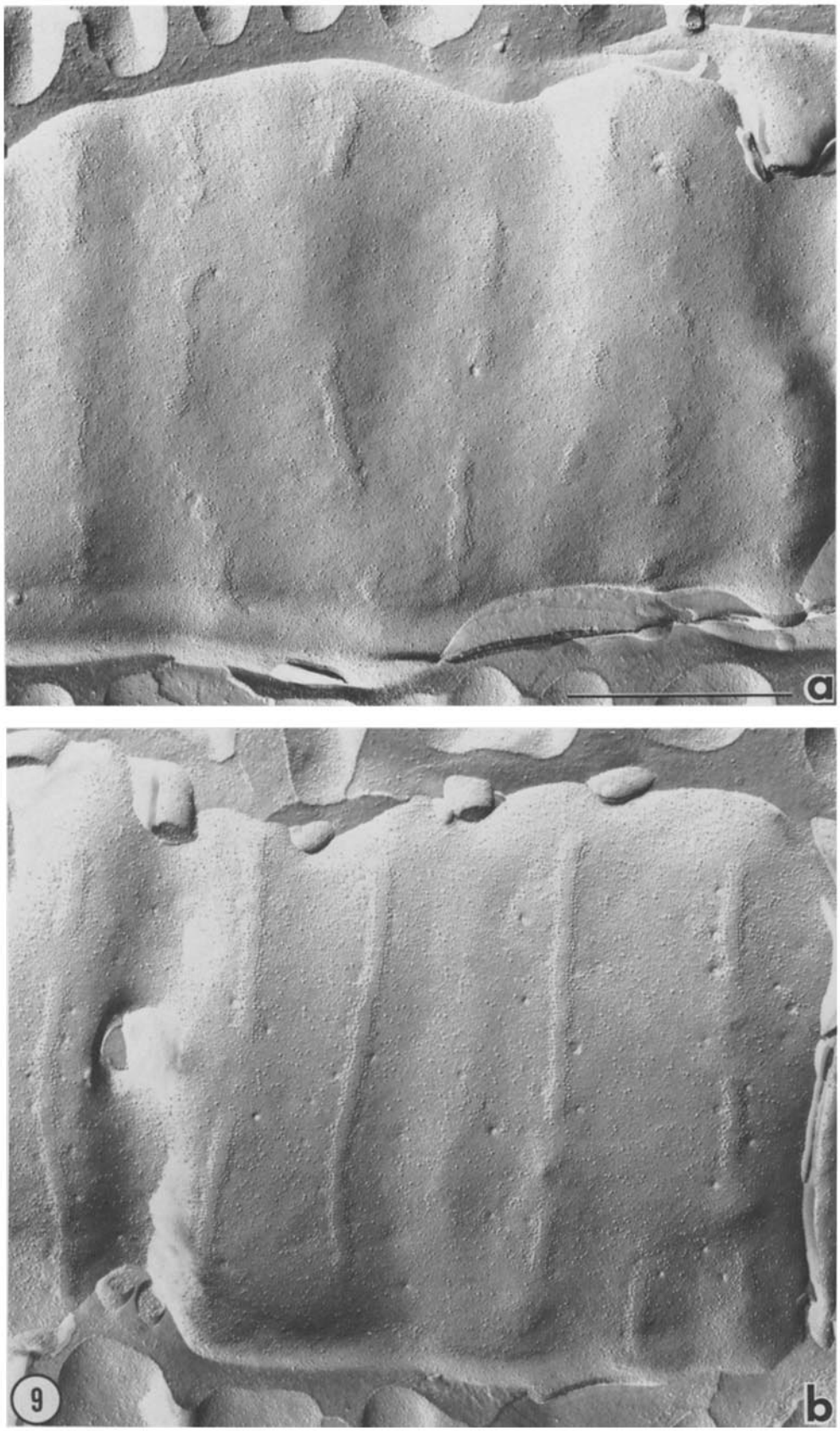


FIGURE 9 Nerve terminal at the elongation stage of regeneration, 78 d pd. *a* and *b* show two regions of the same terminal. The nerve was fixed during stimulation. In *a*, most active zones are still as immature as in the previous stage. In *b*, many active zones appear more organized and are indistinguishable from normal active zones. Bar, 1 μm. × 34,000.

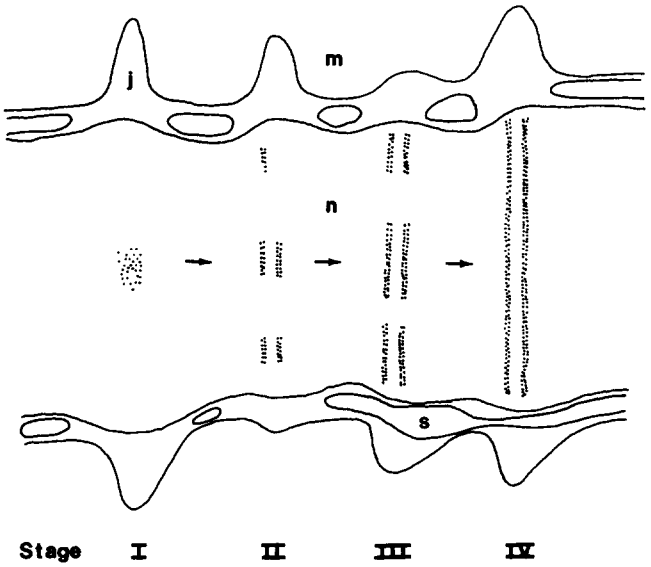


FIGURE 10 A schematic diagram showing the sequence of regeneration of the active zone during stage I (the initial stage); stage II (the secondary stage); stage III (the elongation stage); and stage IV (the late stage). Details in text. Drawings are not to scale. *j*, junctional fold; *m*, muscle fiber; *n*, nerve terminal; *s*, Schwann cell.

particles are observed. These particles are located at specific regions of the terminal membrane, opposite the junctional folds. Even though these particles are not yet organized into the adult pattern of two double rows, they are already functional and mediate exocytosis of synaptic vesicles. Subsequent to forming clusters, active zone particles begin to organize into either one single or one or two double rows with normal orientation. This process occurs primarily during the secondary stage. At this stage every junction becomes functionally reinnervated but active zones still are not completely regenerated. One important feature during this time is the common occurrence of two or more active zone segments lined up at the same junctional fold. Each individual active zone is still short and the total length of active zone per junctional fold is only one-third of normal. At the elongation stage the number of active zone segments remains high. However, individual active zone segments elongate, and hence the total length of active zones per junctional fold increases to two-thirds of normal. In the final stage, these discontinuous active zones seem to join together, which results in a complete recovery of the normal form of active zones in most regions. Heuser (10) reports similar discontinuous active zones during regeneration. It is not known why active zones regenerate by joining several short segments instead of simply elongating a single segment of active zone.

Localization of Regenerating Active Zones

It is important to note that clusters of active zone particles appear at specific regions of nerve terminals, even at the initial stage of regeneration. In freeze-fractured endplates, most postjunctional areas immediately below the nerve terminals are fractured away. Nevertheless, the lateral remnants of junctional folds can usually be seen in most regions of freeze-fractured junctions, and throughout regeneration active zone particles are located opposite to these junctional folds. This process is different from development of active zones in the tadpole (18, 22). At the early stage of development in the tadpole, clusters of active zone particles are observed in random locations on the presynaptic membrane. Later, the characteristic organization of two double rows with a membrane ridge appears. These short active zones still have a random location and orientation. However, as development progresses, the active zones align perpendicular to the long axis of the nerve terminal and become located opposite the junctional folds, as in the adult. One possible explanation for the difference between regeneration and development in the localization of early active zones may be related to the appearance of junctional folds. In the adult animal, junctional folds are present and remain unchanged following degeneration and subsequent regeneration (1, 20, 29); moreover, the distance between neighboring junctional folds also stays the same as shown in this study. On the contrary, during embryonic development of neuromuscular junctions, the initial nerve-muscle contacts are morphologically undifferentiated (7, 19, 21, 23). Thus, it is likely that during the early stage of development, short and randomly oriented active zones precede the development of junctional folds and after junctional folds are formed the final disposition of active zones may then be associated with junctional folds and/or their associated structures (4, 22). Sanes et al. (30) have shown, with thin-section electron microscopy, that regeneration of active zones occurs precisely opposite the basal lamina at junctional folds even in the absence of muscle fibers. Therefore, the basal lamina at junctional folds is apparently an important element in the localization of active zones. The present finding of active zone clusters opposite junctional folds, even during the early stage of regeneration, is consistent with the observations of Sanes et al. (30).

Organization of Regenerating Active Zones

Each dimple at an active zone is thought to represent an opening of a synaptic vesicle undergoing exocytosis and to correspond to the release of one quantum of transmitter (12–14). Even if some dimples at active zones may also represent sites of endocytosis (3), the fact that dimples occur at clusters of active zone particles suggests that during regeneration the primitive active zones are already functional. Other observations also suggest that the normal organization of two double rows per se at the active zone is not essential to the release of transmitter. For example, Ceccarelli et al. (2) have shown that exocytosis can occur at disorganized active zones produced by soaking muscles for 2–3 h in Ca^{2+}-free solutions. It has been suggested that active zone particles represent calcium channels (14, 28). Perhaps the important element in transmitter release is the appearance of active zone particles and their associated structures, regardless of their organizations. The minimum number of active zone particles required for exocytosis of synaptic vesicles is not known. Nevertheless, the close association of dimples and regenerating active zones shown here suggests that the presence of active zone particles and/or their associated structures are essential to transmitter release.

Using thin-section electron microscopy and intracellular recordings, Nakajima and colleagues (24, 25) showed that during development of neuromuscular juctions in tissue culture transmitter release can occur even before the appearance of active zones. In thin sections, however, active zone intramembrane particles cannot be seen. It is possible that during the early developmental stage of synapse formation clusters of active zone particles appear, but the characteristic features

normally seen at active zones in thin section, such as electron-dense tufts, have not developed. It would be interesting to know whether the typical features of active zones seen in thin sections appear only when active zone particles are organized into the typical pattern of two double rows.

In a previous study on degeneration of neuromuscular junctions (16), both disorganization of active zones and impairment of transmitter release mechanisms were observed. However, it is not known if there is a causal relationship between the two. Since the present study showed that the normal organization of active zones is not critical to transmitter release, it is unlikely that the disorganization per se at active zones is the cause of transmission failure during degeneration. Rather, the disorganization of active zones is more likely a result of other degenerative changes which may also cause an impairment in the transmitter release mechanism.

Morphometry of Regenerating Active Zones

In addition to the appearance of dimples, physiological recording indicates that active zones do not have to be organized to be functional. After 4 wk pd, EPPs were recorded from all neuromuscular junctions studied. However, even up to 3 mo pd the immature cluster organization of active zone particles was still observed at some terminals. In addition, the morphometric measurements of active zones depicted their general immaturity even though the nerve terminals were presumably functional. Recently, studies using physiological recording, light microscopy, and thin-section electron microscopy during reinnervation of the cutaneous pectoris muscle in the frog have also shown that physiological and morphological features recover at different rates (6, 8). For example, by 12–16 d after a nerve crush, terminal length reaches a plateau near 90–95% of normal, but the quantal content per unit length has not recovered to this extent. One possible reason for the slower recovery in quantal contents is the immaturity of the active zones. As shown in the present work, the total length of all active zone segments per junctional fold increased with time, but at 4 wk pd the length was only 34% of normal. Even at 78 d pd it increased to 67%, which was still significantly shorter than normal. Ding (8) reported that the quantal content per unit length increased to 67% of normal by 7–12 d and remained essentially at this level even at 280 d pd. On the other hand, DeCino (6) used the same preparations but found full recovery in quantal content per unit length by 22 d pd. It is not clear why their results are different. It would be helpful to know whether there is a correlation between quantal content and morphometry of active zones at physiologically identified neuromuscular junctions.

Conclusion

The present study described the sequence of regeneration of the presynaptic active zone in the frog. In addition, it provided further insights into the nature of the active zone. These results indicate that transmitter release can occur even before active zone particles are organized into the normal pattern of two double rows and thus this organization is not necessary for the active zone to be functional. Furthermore, localization of regenerating active zones, even at the early stage of regeneration, is related to the junctional fold and/or its associated structures.

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