The Journal of Neuroscience, November 1989, 9(11): 3844–3860

Disappearance and Reformation of Synaptic Vesicle Membrane Upon Transmitter Release Observed Under Reversible Blockage of Membrane Retrieval

J. H. Koenig and Kazuo Ikeda

Beckman Research Institute of the City of Hope, Duarte, California 91010

The temperature-sensitive mutant of *Drosophila, shibire*$^{ts-1}$, which is normal at 19°C, but in which endocytosis is reversibly blocked at 29°C, was used to deplete synapses of vesicles by inducing transmitter release while membrane retrieval was blocked. When the synapse was kept at 29°C for 8 min, complete vesicle depletion occurred. However, no compensatory increase in the terminal plasma membrane, either as invaginations or evaginations, was observed. Also, no internalized membranous compartment, such as cisternae or coated vesicles, appeared. No invaginations or outpocketings were seen along the axon between release sites, and no evidence for elongation of the whole axon was found. Thus, the vesicle membrane compartment became unobservable as a result of transmitter release. Depleted synapses were observed by electron microscopy at various times after lowering the temperature, so that the process of synaptic vesicle reformation could be observed. In the first 2–3 min at 19°C, gradually enlarging uncoated invaginations of the plasma membrane were observed. Between 5–10 min at 19°C, these invaginations pinched off to form large cisternae. Newly formed synaptic vesicles were observed associated with these cisternae by an electron-dense material. Between 10–20 min at 19°C, the number of synaptic vesicles increased, while the size of the cisternae decreased. Within 30 min, the full complement of vesicles had reappeared. No involvement of the coated vesicle pathway in synaptic vesicle reformation was observed. The data suggest that synaptic vesicle membrane is dissembled at the time of transmitter release and then is reassembled at sites along the plasma membrane and internalized in the form of large cisternae, from which new vesicles are formed.

The vesicle hypothesis, which has been the most well-accepted explanation for transmitter release for several decades, proposes that transmitter is stored in vesicles and released by exocytosis (del Castillo and Katz, 1955). This process involves the fusion of the vesicle membrane with the terminal plasma membrane at the release site and the opening of the vesicle to expel its contents. Synaptic vesicles which appear to be in the process of exocytosis, and openings in the plasma membrane have been observed at terminal release sites at the time of transmitter release, providing good evidence for this theory (Heuser et al., 1974, 1979; Pumplin and Reese, 1977; Ceccarelli et al., 1979; Torri-Tarelli et al., 1985). An adjunct to this hypothesis is the vesicle recycling hypothesis, which further proposes that after fusion, the vesicle membrane collapses into the terminal membrane (Heuser, 1977; Fesce et al., 1980; Heuser and Reese, 1981) and is subsequently retrieved and recycled into new vesicles (Ceccarelli et al., 1973; Heuser and Reese, 1973; Gennaro et al., 1978).

This latter hypothesis predicts that, in instances where vesicle depletion is caused by procedures such as excessive stimulation, which unbalance the rates of exocytosis (transmitter release) and endocytosis (retrieval of membrane) in favor of the former, most of the vesicle membrane will be found incorporated into the plasma membrane. In instances where the depeleted synapse had previously contained many vesicles, a great deal of additional membrane should be observed in the terminal plasma membrane or other membranous compartments related to recycling, such as coated vesicles or cisternae. Indeed, various researchers have reported such increases in plasma membrane and recycling-related organelles following vesicle depletion (Clark et al., 1972; Ceccarelli et al., 1973; Heuser and Reese, 1973; Pysh and Wiley, 1974; Gennaro et al., 1978; Wiley et al., 1987), although there have been other instances reported where no increases were observed accompanying depletion (Coté et al., 1970; Parducz and Feher, 1970; Birks, 1974; Basbaum and Heuser, 1979; Tremblay and Philippe, 1981).

In the present study, the synapses of the cervical and coxal muscles of *Drosophila melanogaster* were depleted of synaptic vesicles using a temperature-sensitive endocytosis mutant, *shibire*$^{ts-1}$ (*shi*). It is probable that a change in the DNA of a single gene causes a temperature-sensitive instability in an as yet unidentified protein which is involved in the process of endocytosis. At 19°C, this protein functions normally, but due to the amino acid substitution caused by the mutant DNA, it becomes nonfunctional, possibly due to a configurational change in the molecule when the temperature is raised. This results in a very specific blockage of the process of endocytosis. In *shi* neurons, blockage of endocytosis results in the blockage of synaptic vesicle reformation, which in turn causes vesicle depletion when transmitter release, which is unaffected by the mutation, is induced. Thus, it has been observed that if a *shi* neuron is moderately stimulated (0.5 Hz) at 29°C, the number of synaptic vesicles gradually decreases until depletion eventually occurs

Received Dec. 5, 1988; revised May 17, 1989; accepted May 23, 1989.

We wish to thank Ms. Grace Hong, Ms. Laura Dent, and Mr. David Gibbel for their excellent technical help and Ms. Sharyn Webb for her excellent secretarial help. This work was supported by USPHS NIH grants NS18856 and GM35240 and NSF grant BNS-8415920.

Correspondence should be addressed to J. H. Koenig, Division of Neurosciences, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, CA 91010.

(Koenig and Ikeda, 1987; Koenig et al., 1989). This gradual decrease in vesicles/synapse is accompanied by a gradual decrease in the amplitude of the excitatory junction potential (EJP), and a gradual decrease in the frequency of miniature excitatory junction potentials (MEJPs) (Koenig et al., 1983). It has been confirmed that the effect is exclusively presynaptic, so that the decrease in the amplitude of the EJP directly reflects the amount of transmitter released (Koenig and Ikeda, 1983). In an experimental condition where synaptic activity is suppressed while raising the temperature, so that depletion does not occur, transmission is normal at 29°C (Salkoff and Kelly, 1978). Thus, it is assumed that depletion occurs as a result of the synaptic vesicle population having been gradually utilized in the process of transmitter release, without the usual replenishment by endocytosis from the plasma membrane which would normally occur. When the temperature is returned to 19°C, the vesicle reformation process proceeds once more, so that within 30 min, the synapse has recovered its full complement of vesicles, and synaptic transmission is normal (Koenig and Ikeda, 1984).

Since membrane retrieval is blocked at 29°C in this mutant, the vesicle membrane, which is presumably inserted into the terminal plasma membrane upon transmitter release, should remain trapped in the terminal membrane as long as the temperature is raised. Furthermore, since the motor neuron terminals to the cervical and coxal muscles are normally filled with hundreds of vesicles, a terminal depleted of vesicles should exhibit a very significant increase in terminal plasma membrane. However, the present results have shown that in *shi* at 29°C, no significant increase in the terminal plasma membrane, or any other membranous compartment, occurs in the depleted terminals, although a number of small pits were observed on the terminal plasma membrane at this time. Longitudinal sections did not reveal any increase in membrane in between release sites, which are of the *en passant* type, to compensate for this large loss of vesicle membrane.

When the temperature was lowered to 19°C, vesicle reformation began. This process was observed by returning the *shi* fly to 19°C after an initial 8 min exposure to 29°C while stimulating, which depleted the synapses, and noting the condition of the synapses after various exposure times to 19°C, i.e., at various stages of the vesicle reformation process. A gradual increase in the perimeter of the terminal in the form of gradually enlarging uncoated invaginations was observed in this way. These invaginations finally pinched off from the plasma membrane to form cisternae from which synaptic vesicles were remade.

Materials and Methods

The experimental animals used in this study were 4-d-old adult female *Drosophila melanogaster* of the wild-type strain, Oregon-R, and the single-gene mutant, *shi*. Although much of our previous work on *shi* has been done on the dorsal longitudinal flight muscle (DLM) (Ikeda et al., 1976; Koenig and Ikeda, 1983; Koenig et al., 1983; Kosaka and Ikeda, 1983a), the synapses of the ventromedial cervical muscle and the sternal anterior rotator muscle of the first leg (1 of 6 coxal muscles) were chosen for this study because each possessed a particular structural advantage for the experiments. The synapses of the cervical muscle were chosen because they contain many more vesicles than do the DLM synapses. Also, the muscle has a relatively simple innervation pattern by a single motor neuron, whose axon runs along the muscle fiber without branching, making hundreds of *en passant*-type synapses (described in Results). These characteristics are very advantageous for a study of the fate of vesicle membrane during depletion. Intracellular recordings from the cervical muscle (K. Yamaoka and K. Ikeda, unpublished observations) show that these 2 different synapses behave similarly with regard to the effect of the *shi* mutation.

The synapses of the sternal anterior rotator (coxal) muscle were chosen because only a minor dissection is needed to expose the muscle. This dissection is relatively untraumatic to the fly, which is of particular importance in recovery studies. The muscle lies under the median plate of the prothoracic preepisternum of the ventral thorax, attaching medially to the median plate of the sternal apophysis and laterally to the anterior base of the coxa. It is singly innervated by a motor neuron which lies in the prothoracic ganglion and sends its axon through the first leg nerve, which later branches to form the coxal nerve. The coxal nerve reaches the coxal muscle about two-thirds out from the proximal end of the muscle and branches in both directions. The branches run along the outer surface of the muscle at the junctions between the fibers and make hundreds of *en passant* type synapses.

The flies were mounted ventral side up in Tackiwax over an opening in a plastic tube so that when covered with saline (128 mM NaCl; 4.7 mM KCl; 1.8 mM $CaCl_2$; buffered to pH 7.4 with 5 mM Tris aminomethane HCl), the dorsal thorax and entire abdomen, including the spiracles, remained exposed to the air in the tube, allowing the fly to remain in good respiratory condition throughout the experiment. After the fly was embedded in wax and covered with saline, the prothoracic legs were cut with a pair of microscissors, leaving about one-third of the proximal part of the coxa attached to the thoracicocoxal joint, and the ventral thorax was dissected to expose the particular muscle in question. For the cervical dissection, the prothoracic furcosternum was cut by making an incision from the posterior end of one cut coxa to the other. Then, the prothoracic furcum, which is located under the ventral plate of the prothoracic preepisternum along the midline, was cut by a pair of scissors inserted horizontally under the latter through the furcosternal incision. Next, the entire ventral plate was removed by an incision through the anterior end of the bilateral thoracicocoxal joints, keeping the cervical sclerite intact. Thus, the cervical muscles of both sides were exposed to the saline. The cervical nerve was then cut and sucked into a suction electrode for stimulation. Extreme care must be taken with this dissection not to damage the cervical sclerite and the sternal apophysis or the cervical nerve.

For the coxal dissection, the prothoracic furcosternum was cut by making an incision from the posterior end of one cut coxa to the other. Then, cuts were made from the previous incision anteriorly along both lateral edges of the central plate of the episternum up to the anterior edge of the coxal joint where the coxal muscle attaches. As a result of the above dissection, the ventral plate, onto which the coxal muscle attaches medially, is free on 3 sides, posteriorly and laterally, allowing the saline to penetrate under the ventral plate and bathe the coxal muscle. The coxal nerve was then cut and sucked into a suction electrode for stimulation. In this dissection also, extreme care must be taken not to damage the distal attachment of the coxal muscle or the coxal nerve, both of which are located close to the lateral incisions.

After the dissection, the temperature of the saline was raised by instantly replacing the 19°C saline with 29°C saline. The temperature was maintained using a Peltier heating-cooling device and was monitored by a thermistor placed in the bath. During the exposure to 29°C, the nerve was stimulated at 0.5 Hz to deplete the synapses. Eight minutes of stimulation was chosen, because at this point the EJP has disappeared, indicating complete depletion (J. H. Koenig and K. Ikeda, unpublished observations). This particular experimental regime for depleting the synapses differs in 2 respects from our previous experimental regime (Koenig et al., 1983; Kosaka and Ikeda, 1983a). In these previous experiments, (1) the temperature was gradually raised from 19 to 29°C over a period of 5–10 min, then left at 29°C for 10 min; and (2) the synapses were depleted by temperature-induced activity from the thoracic ganglion, which begins at about 26°C (Koenig and Ikeda, 1980). Thus, in our previous experiments, the synapses were depleted between 26–28°C, temperatures at which the blockage of membrane reformation is not as complete as it is at 29°C. This results in some membrane reformation during the depletion process. Also, the synapses were exposed to high temperature for a longer period of time. Since membrane reformation is not completely blocked, even at 29°C, this longer exposure also results in slightly more membrane reformation than an 8 min exposure. Thus, the previous experimental regime resulted in more membrane reformation accompanying depletion than with our present regime (see Discussion for details).

In depletion experiments, after the fly was exposed to 29°C for 8 min, the saline was instantly replaced with fixative (2% paraformaldehyde–

2% glutaraldehyde, in 0.1 M phosphate buffer, pH 7.4). For this purpose, the fixative was poured directly on the exposed muscles. Since the naked terminals are located on the surface of the muscle fibers, fixation must occur very quickly, presumably in the order of milliseconds. In recovery experiments, the temperature of the saline was returned to 19°C after the 8 min exposure to 29°C. After various exposure times to 19°C, e.g., 1, 2, 3, ... 30 min, etc., the saline was instantaneously replaced with the fixative. After fixation for 30 min in the aldehyde mixture, the fixative was replaced with 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 hr. The fly was then postfixed in 2% OsO_4 in 0.1 M cacodylate buffer (pH 7.4), block-stained in 1% aqueous uranyl acetate, dehydrated in alcohol, and embedded in Epon 812. Thin sections of cervical or coxal synapses were observed on a Philips 301 or a Philips CM-10 electron microscope and photographed.

For the depletion experiments, cervical synapses from 47 *shi* flies at 19°C and 43 *shi* flies after 8 min at 29°C were observed and photographed. Ten or more synapses from each fly were observed and photographed, so that over 1000 synapses were observed for this study. Prints of *shi* cervical synapses in both conditions were randomly selected by a technician for measuring. One print from each fly observed at each of the 2 conditions was used for measuring. Thus, each print represented a cervical synapse from a different fly.

For the recovery experiments, coxal synapses from over 200 flies which had been exposed to 29°C for 8 min followed by various intervals of recovery time at 19°C were observed and photographed. Prints of *shi* coxal synapses under the various conditions were randomly selected by a technician for measuring. The perimeters of the cross-sectioned terminals, the synaptic vesicles, and other membranous compartments were measured with the aid of a metric line tracker.

For the light microscopic observations of the cervical innervation, serial sections were impregnated with silver. The detailed methodology can be found in our previous publication (Ikeda et al., 1980). Briefly, the fly was fixed with Bouin's fixative and embedded in paraffin. Serial sections of 8–10 μm were impregnated with silver with our modification of Bodian's protargol method (1936).

Results

Synaptic vesicle depletion

The synapses of the ventromedial cervical muscle were used for these experiments. This muscle is 1 of 8 cervical muscles, but it will henceforth be referred to simply as the cervical muscle in this paper. The muscle lies under the median plate of the prothoracic preepisternum in the ventral thorax, attaching posteriorly to the median plate of the sternal apophysis and anteriorly to the posterior end of the cervical sclerite (Fig. 1*A*). It is singly innervated by a motor neuron which lies in the middle of the cervical connective and sends its axon through the cervical nerve, which later branches to innervate each muscle fiber of this muscle. The innervation of this muscle can be seen in the light micrograph shown in Figure 1. The cervical nerve reaches the cervical muscle about two-thirds out from the anterior end of the muscle. At this point, axons innervating other ventral cervical muscles are separated from the one innervating the ventromedial cervical muscle. The latter reaches the dorsal surface of the ventromedial cervical muscle and makes 6 bifurcations, 1 for each of the 6 muscle fibers composing a ventromedial cervical muscle. Also, at the surface of each muscle fiber, the axon bifurcates making 2 branches which run anteriorly and posteriorly along the muscle fiber (Fig. 1*C*). This is the final branching (Fig. 1*B*). Thus, each muscle fiber is innervated by a single, unbranching axon running parallel to it. The innervation by *en passant* type synapses from a parallel-running single axon characterizes this muscle and is very advantageous for a study of this kind. At a point a few microns away from the final branching, the glial sheath that envelops the axon disappears, so that the axonal membrane is now in direct contact with the muscle fiber membrane. As can be seen in Figure 1, the branches of the axon run along the outer surface of the muscle at the junctions between the fibers. They extend about 100 μm in both directions. At the light microscopic level, the *shi* and wild-type terminals/axon were indistinguishable at any temperature.

A typical electron microscopic longitudinal section of a *shi* cervical axon which is in contact with a muscle fiber at 19°C is shown in Figure 2*A*. As can be seen, release sites occur at intervals of about 1 μm along the axon. The terminal cytoplasm contains many synaptic vesicles, as well as mitochondria. The synaptic vesicles are found almost continuously along the axon. The structure of *shi* terminals at 19°C is indistinguishable from that of wild-type terminals.

In Figure 2, *B, C,* 2 examples of longitudinal sections of *shi* terminals after 8 min exposure to 29°C are shown. As can be seen, the many synaptic vesicles which filled the terminals are now missing. Examples of these terminals at higher magnification in cross section are shown in Figure 3. In Figure 3*A*, an example of a *shi* terminal at 19°C is shown. As can be seen, several release sites are observed within this single plane of sectioning. A release site is characterized by a presynaptic dense body and/or thickening of the pre- and postsynaptic membranes. They often occur onto thin projections of the muscle membrane, as can be seen in this figure. In Figure 3, *B–D,* 3 examples of cross-sectioned *shi* terminals after 8 min exposure to 29°C are shown. Now, the many synaptic vesicles are missing. The mitochondria and presynaptic dense body appear normal. The alignment of the presynaptic dense body with the postsynaptic density is not disrupted. No deep invaginations, unusual outpocketings, or swelling of the terminal are seen, either close to the release sites (Fig. 3, *B–D*) or further along the axon in between release sites (Fig. 2, *B, C*). Also, no internalized membrane compartment, such as coated vesicles or cisternae, is seen. The only abnormality is the appearance of small invaginations of the plasma membrane, which usually possess an electron-dense substance around their neck portions, and have been termed "collared pits" (Kosaka and Ikeda, 1983a). They are usually (>90%) uncoated, although coated-collared pits are also observed. In addition, a few small invaginations without collars were seen, which may represent collared structures in which the collar was out of the plane of sectioning. Occasional small cisterna-like structures are also seen, which usually prove themselves to be enlarged collared pits with serial sectioning. These pits are believed to represent the initial stage of the membrane retrieval process (see Recovery from depletion, below, for full description of coated and uncoated invaginations). Thus, the loss of the vesicle membrane compartment appears not to be accompanied by an equivalent increase in another membranous compartment.

In order to demonstrate this loss of membrane quantitatively, randomly selected electron micrograph prints of cross-sectioned *shi* cervical synapses at 19°C and after 8 min exposure to 29°C were measured for terminal plasma membrane (including coated and uncoated pits, invaginations, and outpocketings) and internalized membrane (coated or uncoated vesicles and cisternae). As can been seen in Table 1, there was no significant increase in the plasma membrane of the terminals at 29°C, the only increase being the few collared pits which appear at this time. However, there was a very significant decrease in the internalized membrane, due to the loss of the synaptic vesicle membrane compartment. As can be seen in Figure 3, *B–D,* very little membrane, other than mitochondrial membrane, is observed in the cytoplasm of these depleted synapses. The 2.1 μm value for cisternal membrane at 29°C in Table 1, line *B* may be

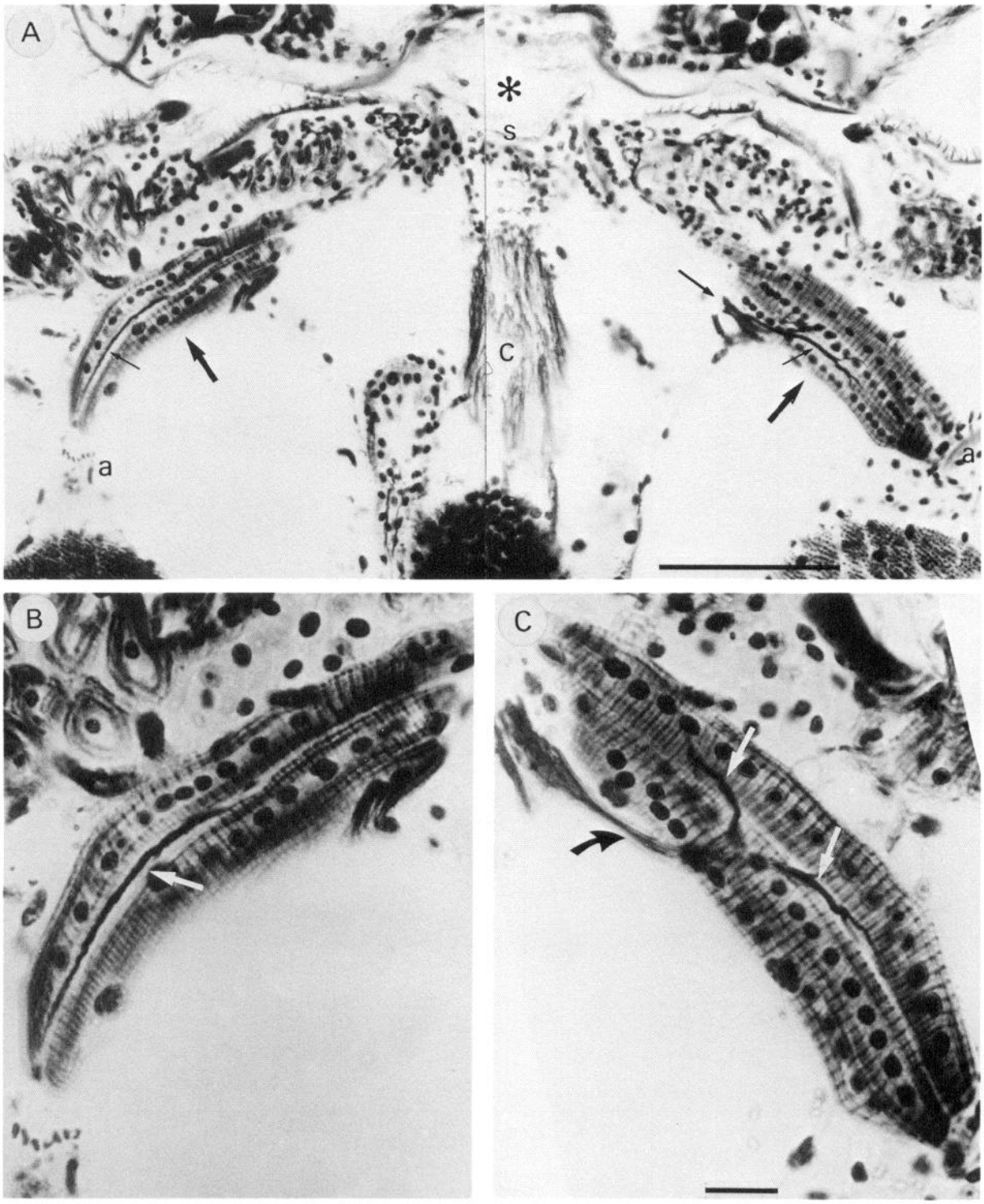


Figure 1. The ventromedial cervical muscle and its innervation. *A,* Horizontal section. The plane of sectioning is tilted with the anterior end upward about 30° (anterior to the *top*). The *asterisk* indicates the space under the neck. The posterior part of the head is seen anteriorly to the asterisk. The structure posterior to the asterisk is the anterior part of the thorax. The ventromedial cervical muscle fibers (*large arrows*) run obliquely from the sternal apophysis (*a*) to the cervical sclerite (*s*). Only the posterior halves of the cervical muscles are seen in this plane of sectioning. The *medium-sized arrow* indicates the cervical nerve approaching the right cervical muscle. The *small arrows* indicate the axons running along the muscle fibers. Scale bar, 50 μm. ×600. Note the bifurcations of the axon on the right to the various fibers. *B,* A cervical muscle shown with higher magnification. The same section as *A*. The *white arrow* indicates a posterior branch of the innervating axon. Note that the axon does not branch. *C,* Anterior–posterior bifurcation. This figure shows a serial section (in the dorsal direction) of the same muscle as that on the right side of *A*. The *white arrows* indicate anterior and posterior branches. The *curved arrow* indicates the axon approaching this cervical muscle. Scale bar, 10 μm. ×1200.

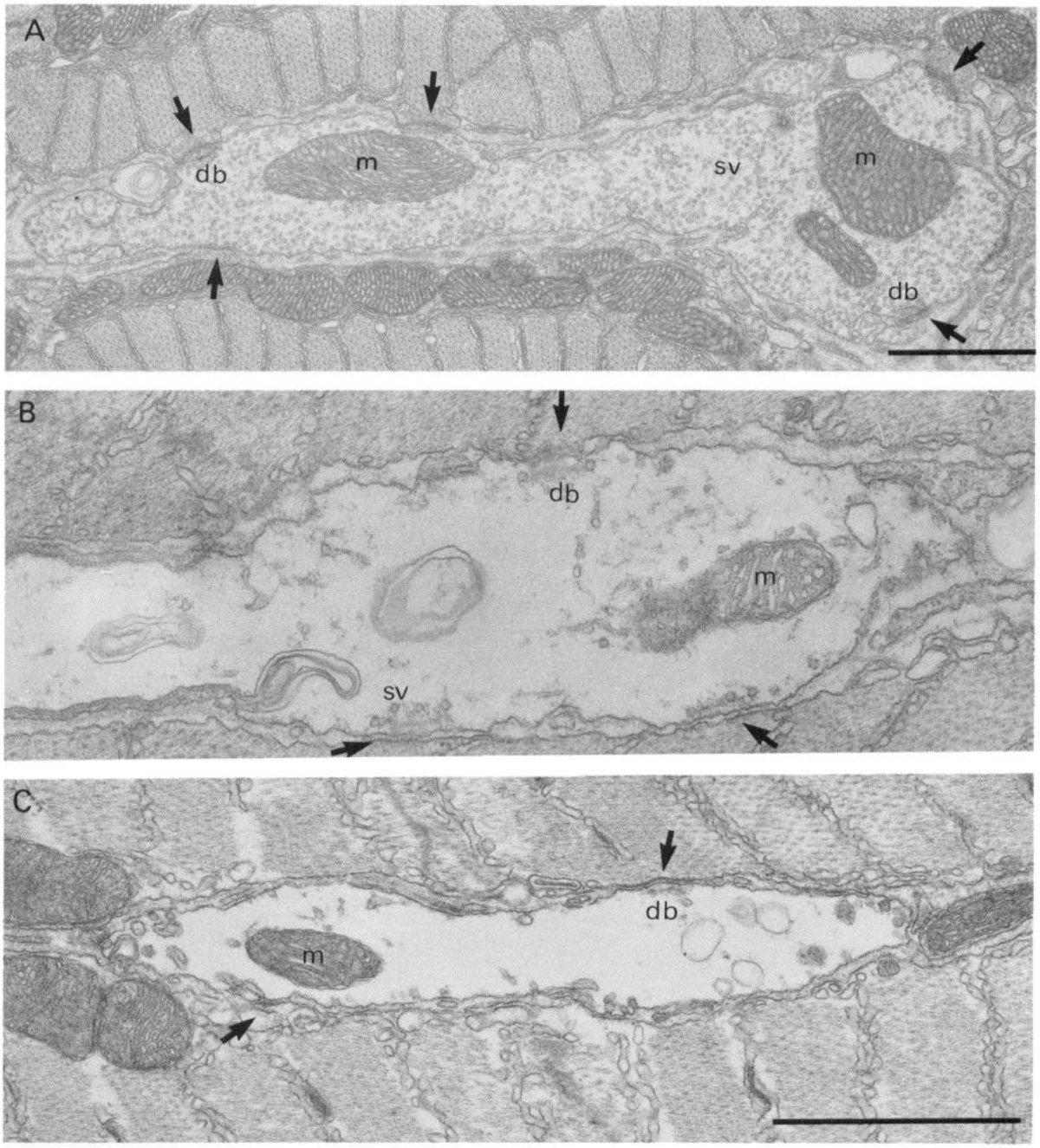


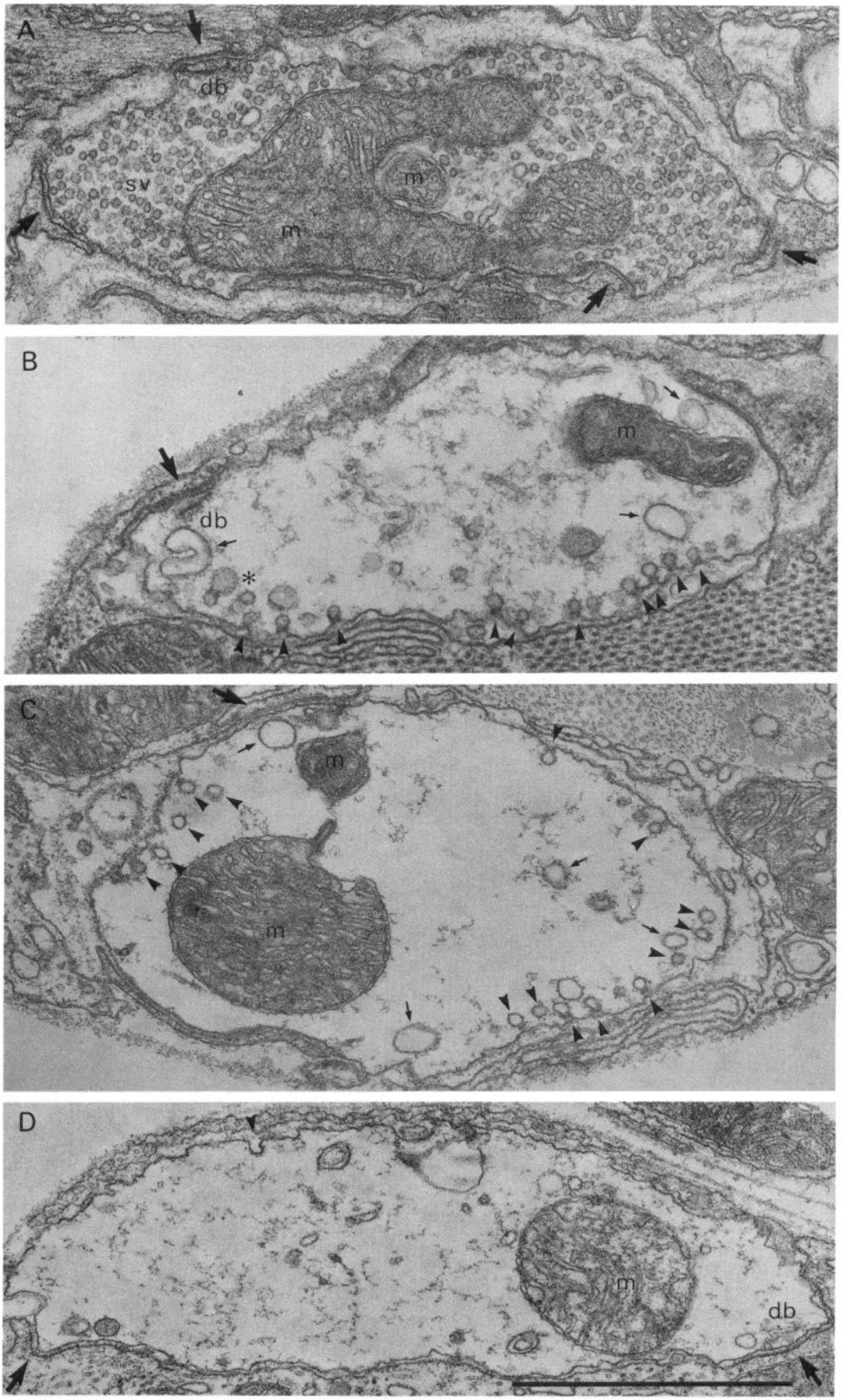
Figure 2. Typical longitudinally sectioned *shi* cervical synapses at 19°C (*A*) and after 8 min exposure to 29°C (*B, C*). Note the loss of many synaptic vesicles and the lack of appreciable increase in other membranous compartments at 29°C. Release sites are designated by *large arrows*. *sv,* synaptic vesicles; *db,* presynaptic dense body; *m,* mitochondria. Scale bar, 1 μm. ×22,750 for *A,* 38,000 for *B* and *C.*

higher than the actual internalized membrane, since any cisternal or vesicle-like structure which was not continuous with the plasma membrane was considered to be internalized, even though these structures may have been continuous with the plasma membrane out of the plane of sectioning. As can be seen in Table 1, line *B,* this loss of vesicle membrane results in a decrease by over 50% in the total observable membrane at 29°C.

The amount of synaptic vesicle membrane in these terminals

→

Figure 3. Typical cross-sectioned *shi* cervical synapses at 19°C (*A*) and after 8 min exposure to 29°C (*B–D*). Note depletion of synaptic vesicles in *B–D* and lack of compensatory increase in the perimeter of the terminal or in other membranous compartments in the cytoplasm. In *B–D,* a number of collared pits are visible along the plasma membrane (*arrowheads*). A few larger invaginations, whose neck portions are not within the plane of sectioning, are also seen (*small arrows*). Release sites, which are characterized by pre- and postsynaptic membrane densities plus a presynaptic dense body, are designated by *large arrows.* In some instances, the dense body is out of the plane of sectioning. The *asterisk* indicates 2 examples of one collared pit growing off of another. Note the thin projections of muscle membrane which encircle the terminal, e.g., in *C. db,* presynaptic dense body; *m,* mitochondria; *sv,* synaptic vesicles. Scale bar, 1 μm. ×47,500 (*A–D*).


A
db
sv
m
m
B
m
db
*
C
m
m
D
m
db

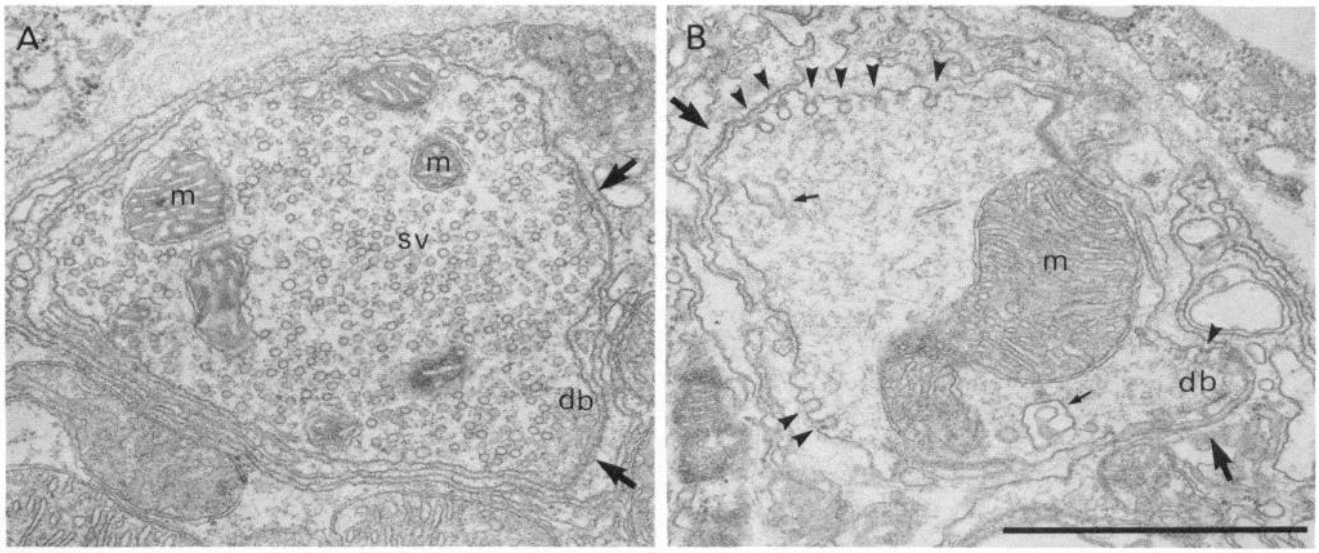


Figure 4. *A,* Typical coxal synapse of a *shi* fly at 19°C, which is characterized by many synaptic vesicles (*sv*), mitochondria (*m*), and a presynaptic dense body (*db*). The release sites, which include pre- and postsynaptic membrane densities plus a presynaptic dense body, are identified by *short, thick arrows. B,* Typical coxal synapse of a *shi* fly after 8 min at 29°C. Note the loss of synaptic vesicles and the appearance of several small invaginations of the plasma membrane usually possessing an electron-dense substance around their neck portions, called collared pits (*arrowheads*). Also note the larger invaginations of the plasma membrane with their neck portions out of this plane of sectioning (*small arrows*). *m,* mitochondria; *db,* dense body. Scale bar, 1 μm. ×40,000.

is very substantial relative to the amount of axonal plasma membrane. Measurements of cross-sectioned terminals show that, in any particular plane of sectioning, the amount of vesicle membrane observed is almost twice that of the plasma membrane which is encircling it (Table 1). If the axon in contact with a muscle fiber membrane, which is approximately 200 μm long, were considered to be a cylinder of 2 μm diameter (estimated from perimeter measurements), then the axonal plasma membrane surface area would be 1256 μm^2. On the other hand, if it is assumed that 200 vesicles of 50 nm diameter are contained in every 100 nm slice along three-quarters of its length (a conservative estimate), then 2400 μm^2 of vesicle membrane would have been added to the axonal plasma membrane in the depleted condition. Although this estimate is quite crude, it points out the enormous expansion of the axon which would occur if the vesicle membrane compartment were inserted into the terminal plasma membrane. No indication of such an increase was observed, however.

Table 1. Temperature-induced changes in membrane compartments of *shi* cervical synapses

Temperature	Average terminal plasma membrane (μm) (SD)	Average synaptic vesicle membrane (μm) (SD)	Average cisternal membrane (μm) (SD)	Average total membrane (μm)
A. 19°C ($N = 47$)	7.05 (4.3)	13.68 (6.1)	0.12	20.85
B. 29°C ($N = 43$)	7.2 (5.4)	0.04	2.1 (1.2)	9.34
C. 19°C (30 min recovery; $N = 40$)	7.0 (3.9)	11.9 (5.8)	1.8 (0.7)	20.7

The blocking effect on recycling at 29°C in *shi* is completely reversible if the temperature is brought down to 19°C. As can be seen in Table 1, after 30 min at 19°C, the *shi* cervical synapses have recovered their full complement of synaptic vesicles. The recovery process is described in detail below using the coxal synapses, which recovered more consistently than did the cervical synapses. This presumably occurred because of the less traumatic dissection necessary to expose the coxal muscle.

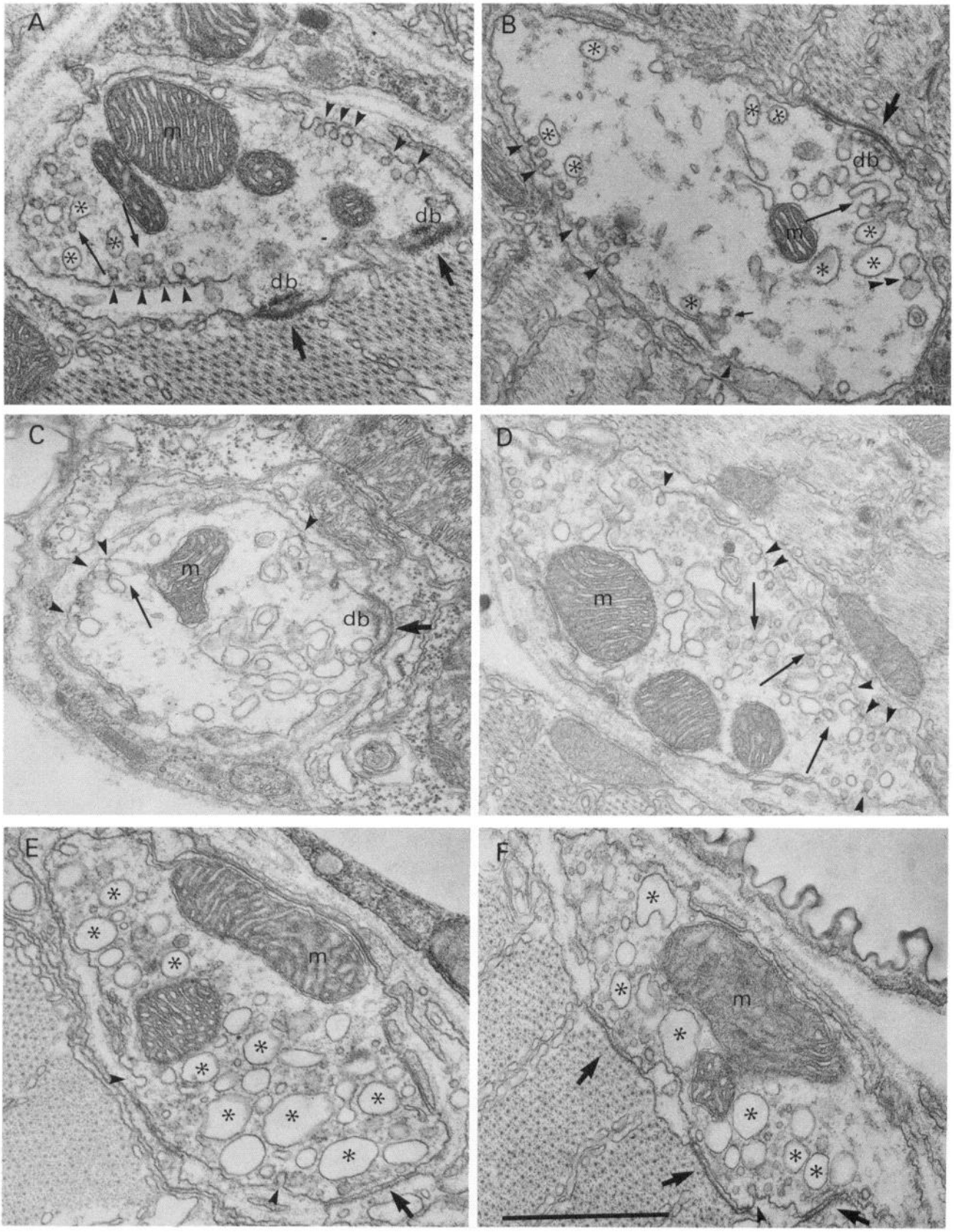
Recovery from depletion

The synapses of the coxal muscle, like those of the cervical muscle, are normally filled with vesicles, as can be seen in the typical coxal synapse shown in Figure 4*A* (*shi,* 19°C). As demonstrated in Table 2, line *A,* the vesicle membrane compartment comprises about 70% of the total observable membrane in a section through a typical coxal synapse. This synapse is morphologically indistinguishable from a wild-type synapse at 19°C. When the *shi* fly is exposed to 29°C for 8 min, vesicle depletion occurs in these synapses, as it occurred in the cervical synapses, and again, no compensatory increase in plasma membrane was observed (Fig. 4*B,* Table 2, line *B*).

When the temperature was lowered to 19°C, the temperature-sensitive blockage of the membrane retrieval process was re-

→

Figure 5. *A* and *B,* Typical coxal synapses of a *shi* fly which was exposed to 29°C for 8 min, followed by 2 min recovery at 19°C. Many collared pits are seen along the plasma membrane (*arrowheads*). Note that the head portions of some of them are enlarged, and in some cases other pits are seen growing off of them (*long, thin arrows*). In *B,* a coated-collared pit is seen growing off of another pit (*short, thin arrow*). Also, the figure-eight-shaped structure in *B* represents one pit growing off of another (*double arrowhead*). The larger cisterna-like structures are probably continuous with the plasma membrane, although in most cases their neck portions are out of this plane of sectioning (*asterisks*). The release sites are identified by *short, thick arrows. m,* mitochondria; *db,* dense body. *C* and *D,* Typical *shi* coxal synapses after 5 min recovery (19°C) from 8 min exposure



to 29°C. Note complex branching invaginations/cisternae in the interior of the terminal, as well as collared pits of varying sizes and complexity at the plasma membrane (*arrowheads*). Also, some collared pits are seen growing off of other pits/cisterna (*long, thin arrows*). *m,* mitochondria; *db,* dense body. *E* and *F,* Typical *shi* coxal synapses after 8 min recovery (19°C) from 8 min exposure to 29°C. Note the many large cisternae (some marked by *asterisks*) in the cytoplasm and the decrease in the number of small pits (*arrowheads*) at the plasma membrane. *m,* mitochondria; *db,* dense body. Scale bar, 1 μm. ×30,000 (*A–F*).

Table 2. Changes in membrane compartments during recovery from depletion in *shi* coxal synapses

	Average perimeter membrane (μm) (SD)	Average cisternal membrane (μm) (SD)	Average pit/ invagination membrane (μm) (SD)	Average vesicle membrane (μm) (SD)	Total observable membrane (μm)
A. Normal state at 19°C (n = 27)	6.40 (1.38)	0.69 (0.85)	0	14.90 (5.56)	21.99
B. Depleted state at 29°C (n = 27)	6.39 (1.59)	1.50 (1.47)	1.23 (0.86)	0	9.12
C. 0–5 min recovery at 19°C (n = 20)	6.13 (1.10)	4.06 (1.84)	2.61 (0.95)	0	12.80
D. 5–10 min recovery at 19°C (n = 19)	6.10 (1.32)	8.48 (3.05)	2.21 (1.50)	0	16.79
E. 10–15 min recovery at 19°C (n = 20)	5.93 (1.40)	6.47 (1.42)	0.27 (0.43)	2.21 (1.43)	14.88
F. 25–30 min recovery at 19°C (n = 18)	6.32 (1.53)	3.92 (3.08)	0	9.05 (5.09)	19.29

leased and recovery from depletion began. The recovery process occurs as a synchronized wave of membrane retrieval beginning at the pit formation stage (Fig. 4*B*, time 0). At various times after bringing the temperature back to 19°C following exposure to 29°C for 8 min, the coxal synapses were observed. Since the retrieval process was occurring in a synchronized manner, the various stages of recovery could be easily discerned as an abundance of specific intermediate structures at any particular time in the recycling process.

Stage 1. Reappearance of membrane (0–10 min recovery at 19°C)

Between 0 and 10 min at 19°C following 8 min exposure to 29°C, coxal synapses exhibited a gradual increase in the total observable membrane, as more collared pits appeared on the terminal membrane and their head portions began to enlarge (Table 2, lines *C, D*). As can be seen in Figure 5, *A, B,* which represents synapses after about 2–3 min recovery at 19°C, the head portions of the pits begin to increase in length and diameter, and also, other pits begin to grow from them, usually possessing a "collar" at the growing point. The uncollared pits also enlarged, and collared pits were seen to branch off of them as well. Thus, a complex network of branching channels which were continuous with the plasma membrane was formed. In Figure 6, the gradual increase in the head portion of the pits is shown at higher magnification. In Figure 6*A*, pits typical of 29°C are seen. Their head portions are similarly sized, approximately 60–80 nm in diameter, and they possess an electron-dense "collar" around their neck portions. With appropriate planes of sectioning, it can be discerned that the collar is actually a tube-like structure which encircles the neck portion of the pits. In Figure 6*A*, the collar (indicated by the short arrow) is sectioned tangentially so that it appears as 2 parallel lines across the neck portion; in Figure 6*B*, the collar (indicated by the short arrow) is seen in cross section, so that it appears as 2 open circles, one on either side of the neck portion. In Figure 6*B*, the very early stages of pit enlargement are seen. As can be seen, the pit head portions have now begun to enlarge, particularly the ones on the right. In Figure 6*C*, the head portions of 2 of the pits have enlarged considerably, while others are of a size more typical of 29°C. The pit on the left appears to be branching. In Figure 6*D*, a pit which has enlarged and branched considerably is seen, and a branch point with an electron-dense collar at its neck portion is also shown. Thus, the branching appears to be accomplished by one collared pit growing from another. The cisterna-like structures, when observed with serial sectioning, are found to be continuous with the extracellular space. For example, the connection between the opening at the plasma membrane in Figure 6*D* and the cisterna-like structures in Figure 5*C*, which is a serial section with Figure 6*D*, can be traced. Also, the electron-dense collar at the neck portion of the branching pit seen in Figure 6*D* is out of the plane of sectioning but can be seen in Figure 5*C*.

In Figure 6, *C, D,* examples of coated profiles (pits/vesicles) can be seen, and the inset in Figure 6*D* shows one of these with higher magnification. Coated profiles were occasionally seen in these recovering synapses, but they represented <5% of the pits seen at this time. Thus, in 50 randomly selected electron micrographs of *shi* terminals, of the 592 pits observed, only 24 were coated. The synapse in Figure 6*D* is therefore unusual, since it includes 3 coated profiles. Such structures were seen to grow off of the plasma membrane or off of uncoated collared pits or invaginations. For example, the coated structure, which is shown at higher magnification in Figure 6*D*, appears to be a coated vesicle but may actually be a coated pit attached to one of the deep invaginations near it. In general, these coated structures were always seen close to the plasma membrane or close to a deep invagination of the plasma membrane. These coated pits also possessed a collar around their neck portions, as is seen, for example, with the coated pit at the upper left in Figure 3*D*.

Thus, the head portions of the pits enlarge and branch so that more and more membrane was observed in the synapses as time went on. This can be seen in Figure 5, *C, D,* which represents synapses after about 3–5 min recovery. At this time, the enlarged pits become complex branching invaginations with a collar at each branch point. This complex structure is shown at higher magnification with serial sectioning in Figure 7, *A–C*. When these sections are superimposed, it can be determined that they all interconnect. Note the electron-dense collars at the branch points. Gradually, fewer and fewer small-diameter collared pits were observed along the terminal membrane, while more and more large cisterna-like structures were seen in the interior of the terminal, as can be seen in Figure 5, *E, F,* which represents about 5–10 min recovery at 19°C. This is also demonstrated in Table 2*D*. Since so few openings (pits/invaginations) of the plasma membrane were seen at this time, as compared with a few minutes less recovery time (Fig. 5, *C, D*), it appears that the deep invaginations have now pinched off from the plasma membrane. Furthermore, the data suggest that pinching off may occur, not only at the plasma membrane, but possibly at each branch point, since the cisternae eventually are not interconnected. This is demonstrated with 3 serial sections in Figure 7, *D–F,* which show many unbranched cisternae in a recovering

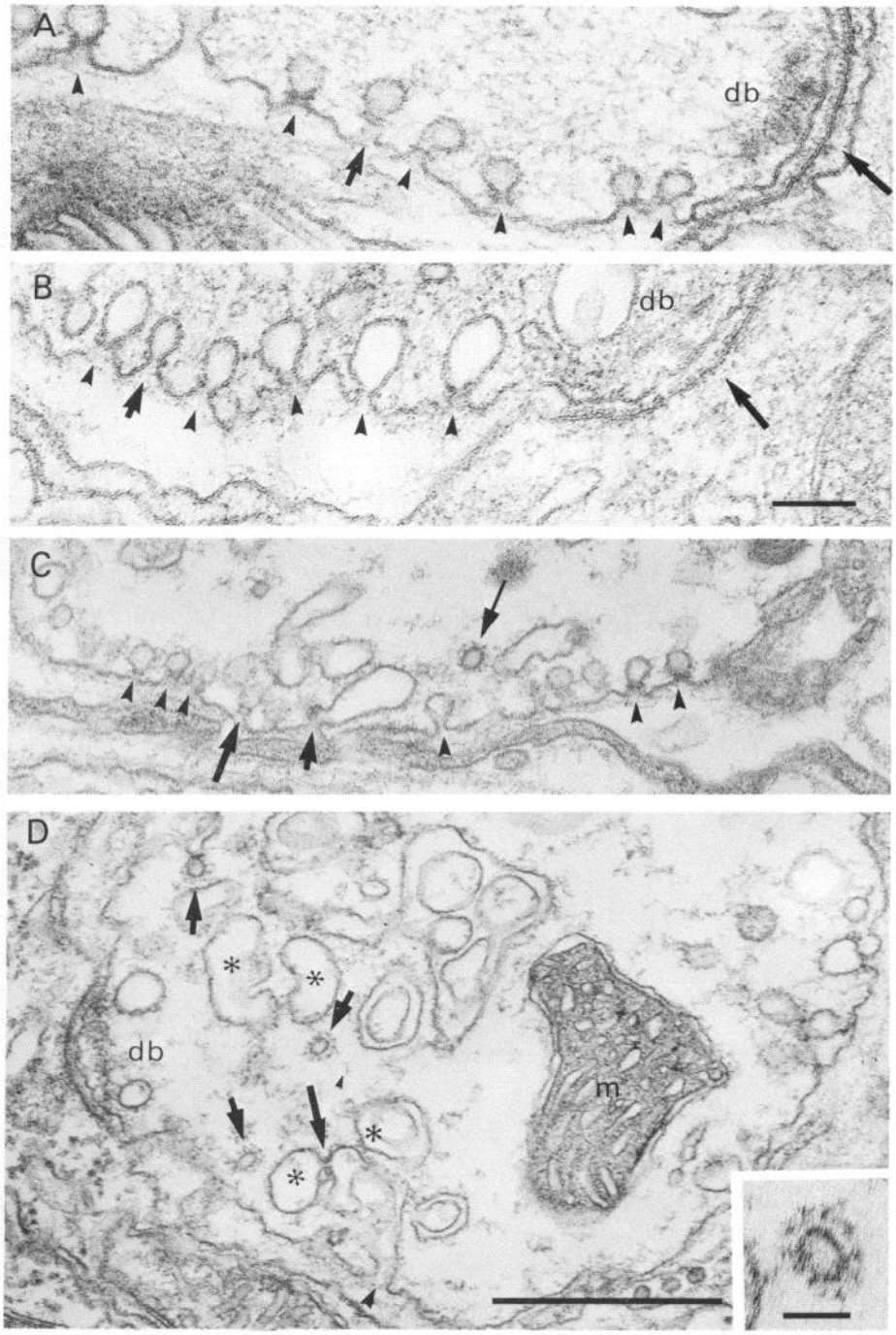


Figure 6. Examples of collared pits in various stages of growth. *A,* Typical collared pits at 29°C (*arrowheads*). Note that the collar of the pit designated by the *short arrow* is seen as 2 parallel lines in this plane of sectioning. *db,* dense body; *long arrow,* release site. *B,* Typical collared pits after about 1 min recovery at 19°C following depletion at 29°C (*arrowheads*). The head portions of the pits, especially the 2 on the right, are somewhat enlarged. Note that the collar of the pit designated by the *short arrow* appears as 2 circular structures, one on either side of the neck portion, in this plane of sectioning. *db,* dense body; *long arrow,* release site. Scale bar, in *B,* 100 nm (for *A* also). ×112,500 (*A, B*). *C,* Typical collared pits (*arrowheads*) after about 2–3 min recovery at 19°C following depletion at 29°C (*arrowheads*). Note 2 enlarged pits designated by *arrows.* The pit on the left (*long arrow*) appears to be branching. A coated profile is pointed out by the *thin-shafted arrow. D,* Typical synapse after about 3–5 min recovery at 19°C following depletion at 29°C. Note the deep pit pointed out by the *arrowhead.* The collar at the neck of this pit/invagination is out of this plane of sectioning but can be seen in Figure 2*C,* which is a serial section with this figure. Another collar is observed at a growing/branch point off of this pit (*long, thick arrow*). Three coated profiles are observed in this synapse (*thin shafted arrows*). The one farthest to the right is shown at higher magnification in the *inset* at the lower right. The cisterna-like structures in the upper-right half of the terminal (2 designated by *asterisks*) are probably also continuous with the plasma membrane, although the opening to the extracellular space is not included in the plane of sectioning. *db,* dense body; *m,* mitochondria. Scale bar, in *D,* 0.5 μm (for *C* also); bar in *inset,* 50 nm. ×62,500 (*C, D*), ×175,000 (inset).

synapse. Thus, during the first 10 min of recovery at 19°C, there is a gradual increase in observable membrane in the terminal in the form of invaginations of the plasma membrane which eventually pinch off to form cisternae. This increase is demonstrated in Table 2, Lines *C* and *D*. As can be seen, a gradual increase in total membrane occurs between 0–10 min recovery, after depletion at 29°C (Table 2, Line *B*), as a result of the increase in pit/cisternal membrane.

Stage 2. Reappearance of synaptic vesicles (10–20 min recovery at 19°C)

After about 10 min recovery at 19°C, synaptic vesicles begin to reappear. These are apparently reformed from the cisternal membrane, which is shown in Figure 5. In Figure 8*A,* an example of a synapse which has partially recovered its complement of synaptic vesicles is shown. Several vesicles are seen hovering close to the dense body. As can be seen, the amount of cisternal membrane has decreased, while the number of vesicles has increased. In particular, note the cisterna closest to the dense body and the synaptic vesicles surrounding it. Such an association between cisternae and vesicles was commonly seen during this stage of recovery. Various examples of this association between cisternae and vesicles are shown in Figure 8, *B–E*. As can be seen in these figures, the cisternae seem to have synaptic vesicles attached to them. Note the electron-dense substance that exists at the point of contact between the cisternal and vesicular mem-

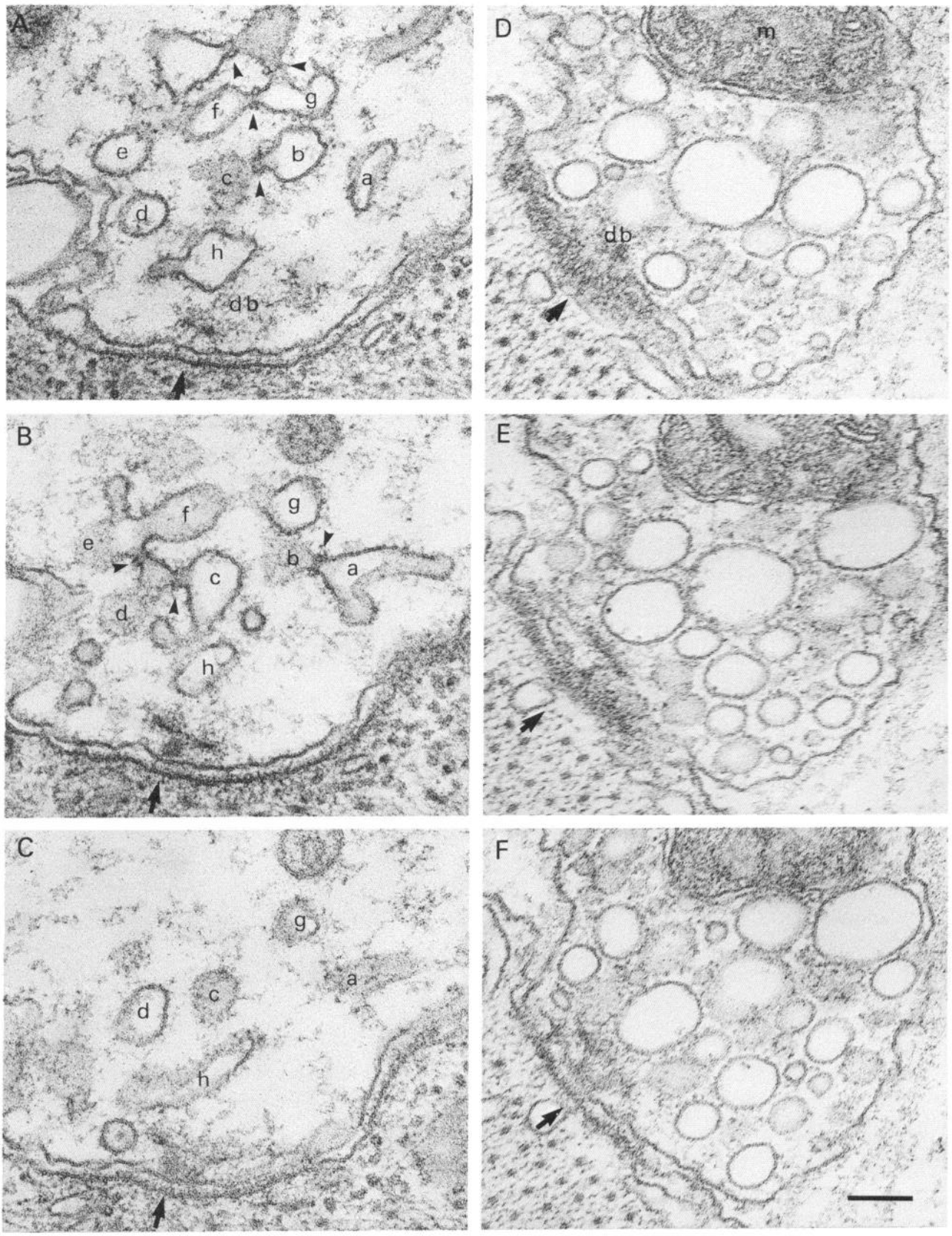


Figure 7. *A–C,* Example of complex branching pattern of the invagination/cisternae which develop after 5–10 min recovery from depletion, shown in 3 serial sections. The small letters (*a–h*) in all 3 sections demonstrate the continuous nature of the cisternae. For example, in *B,* the connection between *a* and *b* is shown. In *A,* the connection between *b* and *c* is shown. In *B,* the connection between *c–f* is shown. In *A,* the connection between *f* and *g* is shown. The *arrowheads* denote the electron-dense collars, which represent the growing point of each branch. *Arrows,* release sites. *D–F,* Examples of cisternae after 10–15 min recovery from depletion. Note that the cisternae are no longer interconnected. *Arrow,* release site; *m,* mitochondria; *db,* dense body. Scale bar, in *F,* 100 nm (for *A–F*). ×112,500 (*A–F*).

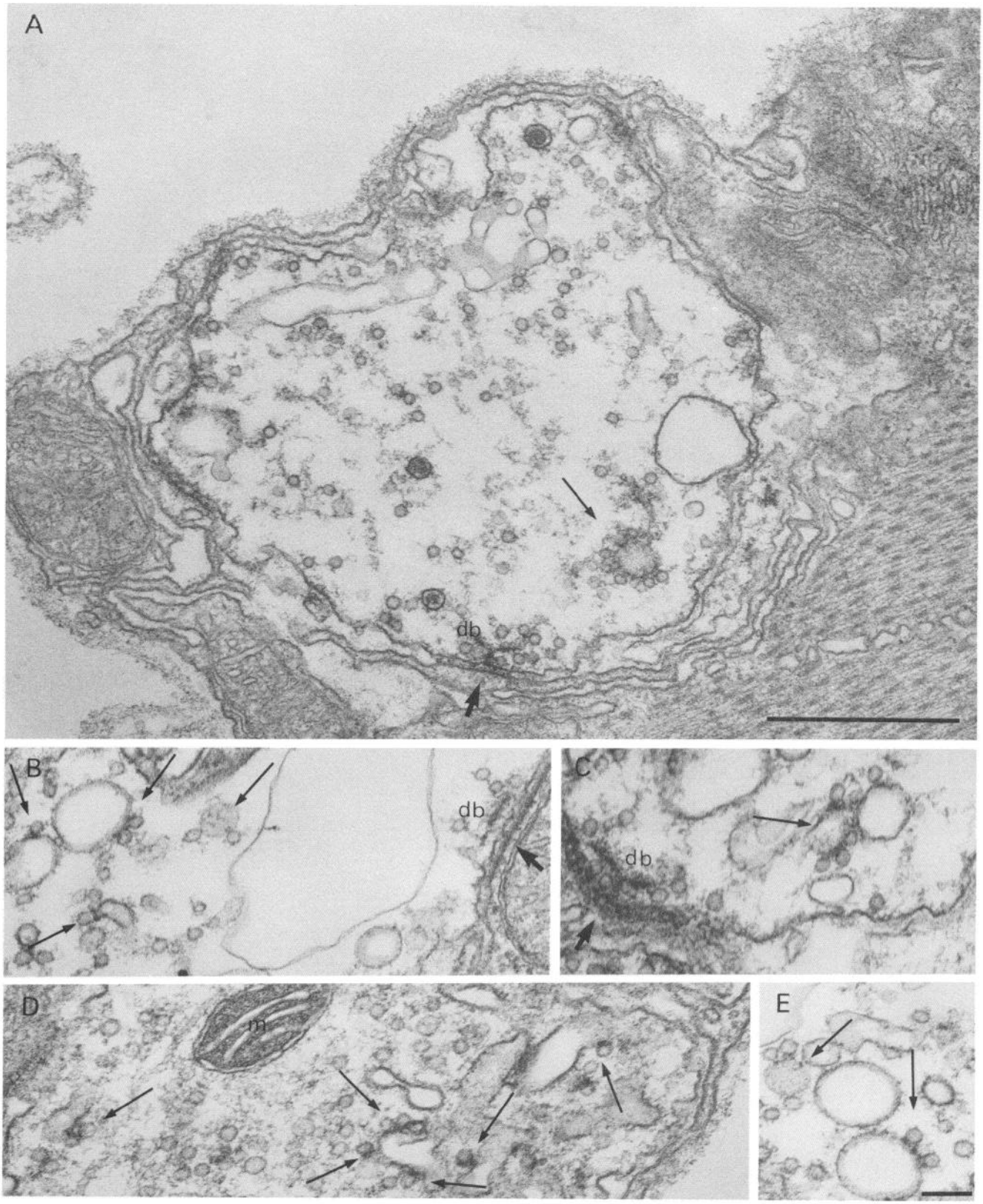


Figure 8. *A,* Typical partially recovered *shi* coxal synapse after 12 min recovery (19°C) from 8 min exposure to 29°C. Note the association of vesicles with the cisternae at *lower right* (*long, thin arrow*). Also, note the association of the vesicles with filamentous material. *m,* mitochondria; *db,* dense body. Release sites designated by *short, thick arrows. B–E,* Higher-magnification examples of the association between newly formed synaptic vesicles and cisternae in recovering coxal synapses (*long, thin arrows*). Note the electron-dense substance at the contact points between the cisternae and the vesicles. Scale bar, in *A,* 0.5 μm; bar in *E,* 100 nm (applies to *B–D* as well). ×68,000 (*A*). ×85,000 (*B–E*).

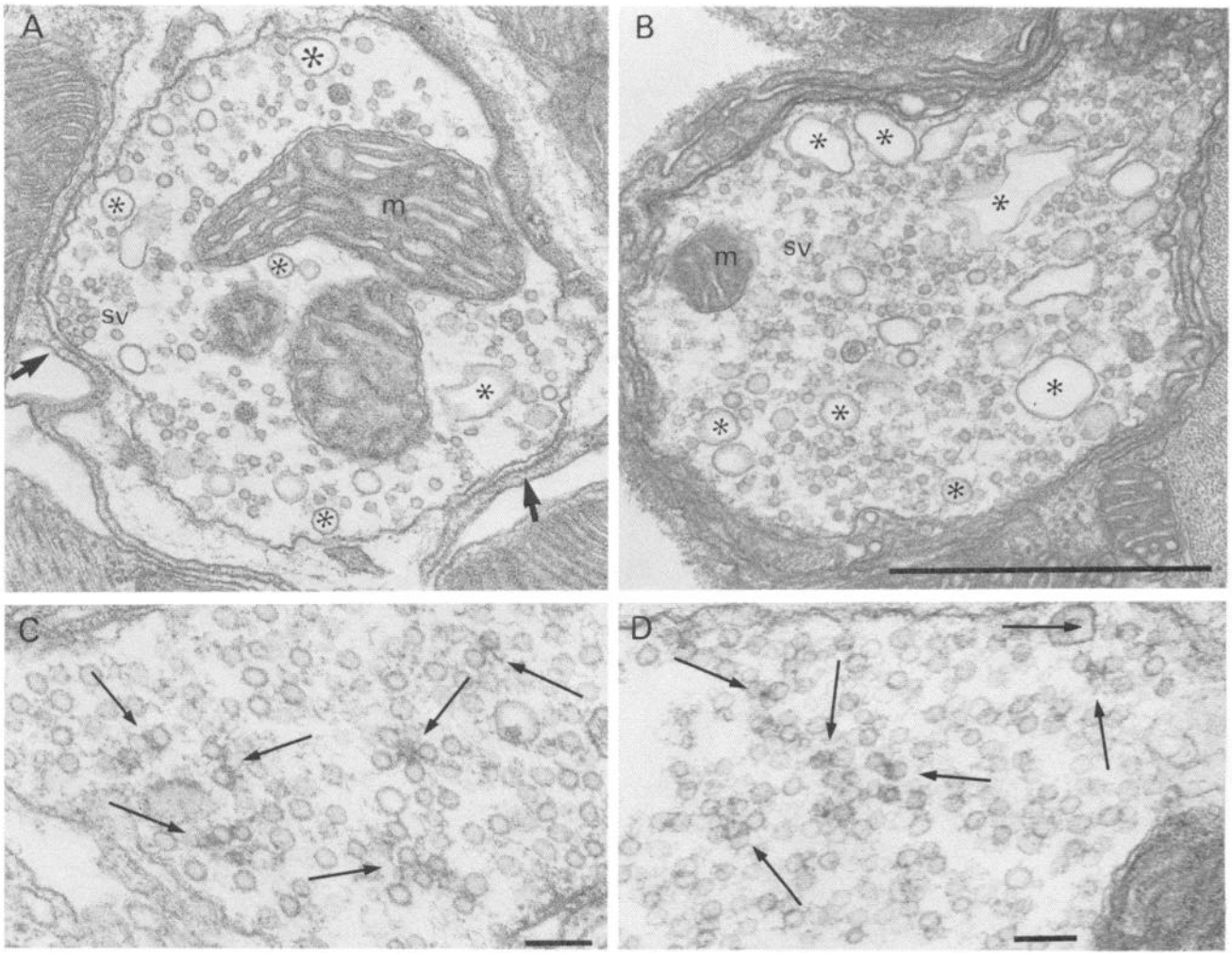


Figure 9. *A* and *B*, Typical examples of *shi* coxal synapses after 25 min recovery (19°C) from 8 min exposure to 29°C. Note the smaller-sized cisternae (some marked by *asterisks*) and many synaptic vesicles (*sv*). The release sites are identified by *short, thick arrows*. *m*, mitochondria. Scale bar in *B*, 1 μm (applies to *A* also). ×45,000 (*A, B*). *C* and *D*, Higher-magnification examples of the electron-dense substance often observed between synaptic vesicles (*long, thin arrows*) at the final stage of recovery. Scale bar, in *C*, 100 nm. ×90,000. Scale bar in *D*, 100 nm. ×85,000.

branes. In some cases, the vesicles seem quite solidly connected to the cisternae by a thick, electron-dense collar. This is seen in many of the associations shown in Figure 8*D*. In other cases, the vesicles seem to have almost dissociated from the cisternae, but are joined by a fine, electron-dense substance. The vesicles/cisternae pointed out by the arrow in the upper left-hand side of Figure 8*E* demonstrate this association. Thus, between 10–20 min recovery, the number of synaptic vesicles increases, while the number and size of the cisternae decrease. This can be seen in Table 2, Line *E*, as a gradual increase in the amount of membrane contributed by synaptic vesicles in synapses recovered for 10–15 min.

Stage 3. Full recovery of synaptic vesicles (20–30 min recovery at 19°C)

Between 20–30 min recovery at 19°C, the coxal synapses were characterized by having many synaptic vesicles, as well as some small-diameter cisternae which tended to be more rounded than were the larger cisternae seen earlier (Fig. 9, *A, B*). Finally, most of the cisternae disappeared, but occasionally an electron-dense material was observed connecting several vesicles, as is shown in Figure 9, *C, D*. The return of vesicles is seen in Table 2, Line *F*.

The stages described above represent a general overview of thousands of observed synapses. The vast majority fit well with the time frame described here. However, exceptions were seen. These involved synapses which seemed to recover more quickly than what is described here or synapses which did not recover at all, or recovered only partially, even after 30 min at 19°C. The speed with which the recovery process occurred was therefore somewhat variable. Possibly, the general well-being of the fly influenced the recovery rate.

Discussion

As mentioned in our introductory remarks, it has been proposed that after transmitter release, synaptic vesicle membrane becomes inserted into the terminal plasma membrane. If the synaptic vesicle membrane were being inserted into the terminal plasma membrane upon transmitter release in *shi* synapses at 29°C, a large increase in the plasma membrane compartment would occur. Measurements of cross-sectioned terminals show

that, in any particular plane of sectioning, the amount of vesicle membrane observed is almost twice that of the plasma membrane which is encircling it. To accommodate this amount of membrane, the terminal plasma membrane could either invaginate into itself, develop outpocketings, or swell generally, so as to increase the length or perimeter of the axon. It can be concluded from the data presented here that no invaginations or outpocketings occur near the release sites or in between them which could account for this enormous amount of membrane. Also, the perimeters of the terminals do not increase. Thus, the only way in which the vesicle membrane could be accommodated in these terminals would be by elongation of the axon. If 2400 μm^2 of vesicle membrane [200 vesicles/100-nm-thick section (see Results, Synaptic vesicle depletion)] were added to the plasma membrane of the existing axon (1256 μm^2), the length of the axon would more than triple. In the opinion of these authors, such an elongation should certainly be observable, especially considering the simple, unbranching structure of this axon/terminal. Certainly, no buckling was seen along the axon between release sites, and no unusual protrusion of membrane was seen at the point where the glial sheath encloses the axon in the cervical nerve. Such an elongation should be very disruptive to the internal organization of the cell, since it would represent an enormous increase in volume, which would probably result in a large uptake of fluid by the cell. Furthermore, it should be quite disruptive to microtubules, which in turn might affect axonal transport. Also, the alignment between the presynaptic dense body and the postsynaptic density might be disrupted. No disruption of this kind was observed. If such disruption occurred, the complete recovery of synaptic function which occurs in *shi* flies would not be expected. Naturally, in a study of this kind, it is not possible to observe the entire cell surface of the neuron or even the entire axon to completely rule out the possibility that expansion might be occurring somewhere else. However, considering the amount of membrane involved, and the relatively straightforward structure of the axon/terminal, the data suggest to these authors that no expansion has occurred.

If no expansion has occurred, then could the plasma membrane accommodate the large amount of vesicle membrane without expanding? This seems unlikely, since no change in the thickness or density of the plasma membrane was observed. Thus, in the opinion of these authors, the most probable explanation for the data is that the vesicle membrane has not been inserted into the terminal plasma membrane by exocytosis, but rather has changed form as a result of transmitter release so as to become unobservable electron microscopically, i.e., it has probably been disassembled.

If one accepts the possibility that the synaptic vesicles at these depleted *shi* terminals have disassembled, then the possibility that this might be the result of an abnormality caused by the *shi* gene must be addressed. This possibility also seems quite unlikely, however, since it has been shown that transmitter release from *shi* terminals is normal at 29°C, if release (which causes depletion) is inhibited while raising the temperature (Salkoff and Kelly, 1978; Koenig and Ikeda, unpublished observations). Also, miniature EJPs are normal at 29°C, although their frequency is greatly reduced (Koenig et al., 1983). Furthermore, it is difficult to imagine how a single gene which codes for a single protein could cause a vesicle membrane disassembly, which is dependent on transmitter release and, in addition, provide the cell with a mechanism for reassembly. Thus, it is most likely that the *shi* gene affects only the membrane retrieval mechanism as has been previously suggested.

These observations may appear to be in direct opposition to those which report increases in other membranous compartments (terminal plasma membrane, coated vesicles, cisternae) accompanying vesicle depletion. However, the discrepancy may be related to the method of depletion used. In the instances where the method used to cause depletion was to greatly increase the rate of transmitter release relative to the rate of membrane retrieval, it might be expected that membranous compartments related to vesicle reformation would be observed, since endocytosis was proceeding during the depletion process in these experiments, possibly even at an accelerated rate. As a matter of fact, in our previous experiments, in which the *shi* synapses were depleted at temperatures between 26–28°C (see Materials and Methods for description of experimental regime) so that the membrane reformation process was not completely blocked during depletion, pits with enlarged head portions and cisternae-like structures were observed in some of the depleted synapses. These terminals looked similar to the synapses shown in various previous reports which observed increases in terminal membrane accompanying depletion, and this led us initially to assume that synaptic vesicle membrane was being inserted into the plasma membrane by exocytosis at the time of transmitter release (Koenig et al., 1983; Kosaka and Ikeda, 1983a). Only when these synapses were depleted at 29°C, when the membrane reformation process is almost completely blocked, did it become apparent that the synaptic vesicle membrane did not disappear *simultaneously* with the appearance of pits/invaginations of the plasma membranes or cisternae. Thus, the balance between depletion and the extent of membrane reformation becomes critical. Apparently, in *shi* synapses at 29°C, the reformation process is sufficiently retarded so that only very small pits form after 8 min, and when transmitter release is stimulated, it produces an uncoupling in time between vesicle depletion and membrane reformation.

The possibility that synaptic vesicle membrane is reassembled at sites along the plasma membrane, rather than inserted into the plasma membrane by exocytosis at the time of transmitter release, is also suggested by our observations on the vesicle reformation process. Thus, the results describe the gradual increase in terminal plasma membrane during recovery from depletion, as the small collared pits seen at 29°C begin to enlarge and branch. Within 10 min at 19°C, the terminal, which was empty at 29°C, is filled with cisternae, which are now for the most part no longer continuous with the plasma membrane. It appears that these cisternae are actually elongated collared pits which have pinched off from the plasma membrane. The electron-dense tube-like structures surrounding the neck portion of the pits may be involved in the pinching-off process which later transforms the deep invaginations into cisternae, since they represent points of constriction where the 2 opposing membranes are brought in close alignment, a requirement for fusion. Also, these "collars" may be involved in the assembly process, since a "collar" is usually observed at each branching point of the elongated pit (see Figs. 6*D* and 7, *A–C*), as well as at the neck of the initial pit on the plasma membrane.

After about 10 min recovery at 19°C, synaptic vesicles begin to reappear in the terminals. At this stage in the recovery process, the vesicles are often seen attached to the cisternae by an electron-dense substance, which may be involved in the pinching-off process. The unattached vesicles in these partially recovered

synapses are often associated with a fine, filamentous substance which seems to interconnect them (see Fig. 8*A*). This interconnecting filamentous substance is less obvious when the synapse is packed with vesicles, but it can be observed even in a normal synapse. Interconnecting filaments have also been reported between synaptic vesicles in *Xenopus* synapses (Smith, 1988). It is interesting to speculate that the electron-dense substance, which lies between the cisternal and vesicular membranes, might unravel as the vesicle pinches off from the cisterna, creating a filamentous link between the cisterna and other vesicles.

It was observed that as more and more vesicles reappeared in the synapses, the size and number of the cisternae decreased, which suggests that the vesicles may actually be made by pinching off of the cisternal membrane. Vesicles which seemed attached to each other by an electron-dense substance were often observed at the final recovery stage, when the cisternae were almost gone (Fig. 9, *C, D*).

These data agree with certain aspects of previously proposed recycling pathways and disagree with other aspects. The proposal of Heuser and Reese (1973) that synaptic vesicle membrane is retrieved from the plasma membrane via coated vesicles which then coalesce to form cisternae does not agree with the present results. No substantial increase in coated profiles was observed as a result of depleting the synapses of vesicles. The possibility that our fixation techniques destroy the delicate clathrin coat seems unlikely, since coated structures were seen, along with uncoated ones, even in the same synapse. Furthermore, even if our fixation techniques were faulty, the fact that the pits elongate and branch, rather than pinch off and fuse to form cisternae, makes the possibility that they are poorly preserved coated pits unlikely.

The *shi* gene affects membrane retrieval of coated as well as uncoated pits. This is shown by observations on the non-neuronal garland cell, which takes up waste products from the haemolymph, and on the oocyte, which is active in the uptake of vitellogenin via coated pits/vesicles. In these cell types, also, the *shi* gene inhibits membrane retrieval, but in these instances, it is retrieval of coated structures. As a result, there is a build-up of coated pits on the plasma membrane of these cells at 29°C (Kosaka and Ikeda, 1983b; Ikeda et al., 1987). Thus, if the coated vesicle pathway were involved in vesicle recycling, it should have been stimulated by the temperature-induced depletion, and many coated pits should have been observed along the plasma membrane at 29°C. However, a build-up of uncoated pits occurred, while very few coated ones were seen.

The lack of coated structures in these depleted synapses is quite different from many reports in the literature, which describe increases in coated profiles as a result of inducing vesicle depletion. Why coated profiles increased in these synapses and not in the ones described here is not known. Possibly, the difference may be related to the methods used to cause depletion. Methods which induce massive transmitter release, such as 4-aminopyridine (4-AP), toxins, or excessive stimulation, may stress the metabolism of the neuron in such a way as to induce coated vesicle production. The method of depletion used here, which employs very moderate release, apparently does not stimulate this coated vesicle pathway. Thus, it appears from these results that the coated vesicle pathway so often observed accompanying vesicle depletion, may not actually be related to vesicle recycling. Certainly, our data show that a full complement of synaptic vesicles can be produced in these synapses without the involvement of coated vesicles. Various other researchers have also suggested that coated vesicles may not be involved in the recycling of synaptic vesicles (Basbaum and Heuser, 1979; Ceccarelli et al., 1979; Kadota and Kadota, 1982; Meshul and Pappas, 1984; Torri-Tarelli et al., 1987). From our observations of the neuron, which show no involvement of coated vesicles in membrane retrieval, as well as of the oocyte and the garland cell, both of which show coated vesicles functioning for the uptake of macromolecules, it appears to us most likely that the coated vesicle pathway is used specifically for the uptake of macromolecules but not for membrane retrieval.

The suggestion that a cisterna might be formed by the coalescence of vesicles was also not supported by these data. Figure-eight-shaped structures such as those seen in Figure 2*B*, which might be interpreted as one small-sized cisterna coalescing with another, were often observed, but serial sectioning revealed that these actually represent one pit branching off from another pit. Also, an electron-dense collar, which is known to occur at the growing point of these pits, was usually seen at the point of junction between the 2 pits, conclusively identifying them as one pit branching off of another. Similarly, it has been suggested that what appear to be images of coated vesicles coalescing with cisternae actually represent coated pits on deep invaginations of the plasma membrane (Gennaro et al., 1978), something which we observed also.

Thus, the results suggest that the first stage of vesicle recycling is via uncoated pits, which do not pinch off and coalesce, but rather elongate and finally pinch off to form cisternae. This pathway conforms more closely with a second proposed recycling pathway in which large invaginations of the plasma membrane pinch off to form cisternae (Miller and Heuser, 1984). Miller and Heuser suggest that this recycling pathway may be abnormal—the result of the massive amounts of vesicle membrane that are added to the plasma membrane as a result of the application of 4-AP. However, our data would suggest that this pathway is actually the major pathway for synaptic vesicle recycling, while the coated vesicle pathway may be involved in something else.

Large invaginations/cisternae in stimulated synapses of the cat sympathetic ganglion have also been reported by Kadota and Kadota (1982). These authors suggest that the cisternae observed in these terminals are transformed into tubular structures or multivesicular bodies and migrate from the terminal area without being recycled into synaptic vesicles. The present observations show a definite association of synaptic vesicles with these endocytotic cisternae, however. Also, retrograde migration of the cisternal membrane as tubules or multivesicular bodies up the axon was not seen, although both crescent-shaped tubules and multivesicular bodies were sometimes observed in these synapses. It has been further proposed that synaptic vesicles bud off of terminal smooth endoplasmic reticulum (SER) which is continuous with axonal SER (Teichberg and Holtzman, 1973; Kadota and Kadota, 1985). It seems unlikely that synaptic vesicles originate from SER in our neuromuscular junctions, however, since these synapses become rapidly depleted of vesicles as a result of blocking membrane retrieval during transmitter release, which should not occur if synaptic vesicles were being manufactured from SER inside the terminal independent of the endocytotic process. Also, in our experiments, the coxal nerve was cut, but recovery from depletion occurred, eliminating the possibility that substances or membrane which are synthesized in the soma and thus must travel down the axon are involved in the recovery process.

Another proposal for membrane retrieval is that after releasing their contents by exocytosis, synaptic vesicles immediately pinch off from the terminal membrane intact, without collapsing into the terminal membrane and mixing with it (Ceccarelli et al., 1972, 1973). Although this proposal does not fit entirely with our observations, the possibility that the vesicle membrane disintegrates immediately after exocytosis, rather than pinching off intact, would be compatible. Our data would also agree with the idea that no mixing of vesicle and plasma membrane occurs. This has been suggested by experiments which show no incorporation of labeled terminal membrane into newly formed vesicle membrane following transmitter release (Lentz and Chester, 1982). The mechanism whereby vesicle membrane, which is of different composition from plasma membrane, disperses into the plasma membrane and is then somehow reconstituted in coated vesicles has been difficult to envision. Reassembly of this differently composed membrane on sites along the plasma membrane, on the other hand, eliminates the problems which are inherent in mixing of membranes.

It has recently been proposed that vesicles may not exocytotically release their contents into the synaptic cleft, but rather disintegrate intracytoplasmically in the immediate vicinity of the presynaptic membrane (Gonzalez-Aguilar et al., 1988). This was proposed as a result of observations that synapses processed by fast chemical fixation showed no exocytotic images during transmitter release, but rather showed vesicles transforming into amorphous electron-dense material. On the other hand, with slower fixation techniques, many exocytotic-like images were observed. Our own electron microscopic observations on synaptic vesicles associated with the release site in coxal synapses also suggest a disintegration process (J. H. Koenig and K. Ikeda, unpublished observations). Certainly, the possibility that release is not accomplished by exocytosis is compatible with the observations presented here, although exocytosis is not ruled out on the basis of these data, since the vesicle disassembly could occur after transmitter release by exocytosis.

Although the mechanisms for disassembly and reassembly of vesicle membrane are in the realm of speculation at the present time, it is hoped that these observations may provide impetus for further research along these lines in the future.

References

Basbaum, C. B., and J. E. Heuser (1979) Morphological studies of stimulated adrenergic axon varicosities in the mouse vas deferens. J. Cell Biol. *80:* 310–325.

Birks, R. I. (1974) The relationship of transmitter release and storage to fine structure in a sympathetic ganglion. J. Neurocytol. *3:* 133–160.

Bodian, D. (1936) A new method for staining nerve fibers and nerve endings in mounted paraffin sections. Anat. Rec. *65:* 89–97.

Ceccarelli, B., W. P. Hurlbut, and A. Mauro (1972) Depletion of vesicles from frog neuromuscular junctions by prolonged tetanic stimulation. J. Cell Biol. *54:* 30–38.

Ceccarelli, B., W. P. Hurlbut, and A. Mauro (1973) Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. J. Cell Biol. *57:* 499–524.

Ceccarelli, B., F. Grohovaz, and W. P. Hurlbut (1979) Freeze-fracture studies of frog neuromuscular junctions during intense release of neurotransmitter. II. Effects of electrical stimulation and high potassium. J. Cell Biol. *81:* 179–192.

Clark, A. W., W. P. Hurlbut, and A. Mauro (1972) Changes in the fine structure of the neuromuscular junction of frog caused by black widow spider venom. J. Cell Biol. *52:* 1–14.

Coté, M. G., D. Palaic, and J. C. Panisset (1970) Changes in the number of vesicles and the size of sympathetic nerve terminals following nerve stimulation. Rev. Can. Biol. *29:* 111–114.

del Castillo, J., and B. Katz (1955) La base "quantale" de la transmission neuromusculaire. In *Microphysiologie Comparée des Éléments Excitables,* Vol. 67, pp. 245–258, Centre National de la Recherche Scientifique, Colloques Internationaux, Paris.

Fesce, R., F. Grohovaz, W. P. Hurlbut, and B. Ceccarelli (1980) Freeze-fracture studies of frog neuromuscular junctions during intense release of neurotransmitter. III. A morphometric analysis of the number and diameter of intramembrane particles. J. Cell Biol. *85:* 337–345.

Gennaro, J. F., W. L. Nastuck, and D. T. Rutherford (1978) Reversible depletion of synaptic vesicles induced by application of high external potassium to the frog neuromuscular junction. J. Physiol. (Lond.) *280:* 237–247.

Gonzalez-Aguilar, F., J. A. Rodriguez, H. Alzola, and M. C. Lupidio (1988) Synaptic vesicle relationships with the presynaptic membrane as shown by a new method of fast chemical fixation. Neuroscience *24:* 9–17.

Heuser, J. E. (1977) Synaptic vesicle exocytosis revealed in quick-frozen frog neuromuscular junctions treated with 4-aminopyridine and given a single electrical shock. Neurosci. Symp. *2:* 215–239.

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

Heuser, J. E., and T. S. Reese (1981) Structural changes after transmitter release at the frog neuromuscular junction. J. Cell Biol. *88:* 564–580.

Heuser, J. E., T. S. Reese, and D. M. D. Landis (1974) Functional changes in frog neuromuscular junctions studied with freeze-fracture. J. Neurocytol. *3:* 109–131.

Heuser, J. E., T. S. Reese, M. J. Dennis, J. Jan, L. Jan, and L. Evans (1979) Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. J. Cell Biol. *81:* 275–300.

Ikeda, K., S. Ozawa, and S. Hagiwara (1976) Synaptic transmission reversibly conditioned by single-gene mutation in *Drosophila melanogaster.* Nature *259:* 489–491.

Ikeda, K., J. H. Koenig, and T. Tsuruhara (1980) Organization of identified axons innervating the dorsal longitudinal flight muscle of *Drosophila melanogaster.* J. Neurocytol. *9:* 799–823.

Ikeda, K., T. Tsuruhara, and J. H. Koenig (1987) A study of endocytotic pathway in the oocyte using a temperature sensitive mutant of *Drosophila melanogaster.* J. Cell Biol. Abstr. *105:* 230a.

Kadota, T., and K. Kadota (1982) Membrane retrieval by macropinocytosis in presynaptic terminals during transmitter release in cat sympathetic ganglia *in situ.* J. Electron Microsc. *31:* 73–80.

Kadota, T., and K. Kadota (1985) Tubular network of the smooth endoplasmic reticulum which appears in the axon terminal following stimulation of the cat sympathetic ganglion *in situ.* Biomed. Res. *6:* 13–22.

Koenig, J. H., and K. Ikeda (1980) Flight pattern induced by temperature in a single-gene mutant of *Drosophila melanogaster.* J. Neurobiol. *11:* 509–517.

Koenig, J. H., and K. Ikeda (1983) Evidence for a presynaptic blockage of transmission in a temperature sensitive mutant of *Drosophila.* J. Neurobiol. *14:* 411–419.

Koenig, J. H., and K. Ikeda (1984) Synaptic vesicle recycling studied using an endocytosis mutant. Soc. Neurosci. Abstr. *1:* 873.

Koenig, J. H., and K. Ikeda (1987) Synaptic vesicle number correlated with EJP amplitude. Soc. Neurosci. Abstr. *13:* 317.

Koenig, J. H., K. Saito, and K. Ikeda (1983) Reversible control of synaptic transmission in a single gene mutant of *Drosophila melanogaster.* J. Cell Biol. *96:* 1517–1522.

Koenig, J. H., T. Kosaka, and K. Ikeda (1989) The relationship between the number of synaptic vesicles and the amount of transmitter released. J. Neurosci. *9:* 1937–1942.

Kosaka, T., and K. Ikeda (1983a) Possible temperature-dependent blockage of synaptic vesicle recycling induced by a single gene mutation in *Drosophila.* J. Neurobiol. *14:* 207–225.

Kosaka, T., and K. Ikeda (1983b) Reversible blockage of membrane retrieval and endocytosis in the garland cell of the temperature-sensitive mutant of *Drosophila melanogaster, shibire*^{ts}. J. Cell Biol. *97:* 499–507.

Lentz, T. L., and J. Chester (1982) Synaptic vesicle recycling at the neuromuscular junction in the presence of a presynaptic membrane marker. Neuroscience *5:* 691–718.

Meshul, C. K., and G. D. Pappas (1984) The relationship of pino-

cytosis and synaptic vesicles at the frog neuromuscular junction. Brain Res. *290:* 1–18.

Miller, T. M., and J. E. Heuser (1984) Endocytosis of synaptic vesicle membrane at the frog neuromuscular junction. J. Cell Biol. *98:* 685–698.

Parducz, A., and O. Feher (1970) Fine structural alterations of presynaptic endings in the superior cervical ganglion of the cat after exhausting pre-ganglionic stimulation. Brain Res. *362:* 375–378.

Pumplin, D. W., and T. S. Reese (1977) Action of brown widow spider venom and botulinum toxin on the frog neuromuscular junction examined with freeze-fracture technique. J. Physiol. (Lond.) *273:* 443–457.

Pysh, J. J., and R. G. Wiley (1974) Synaptic vesicle depletion and recovery in cat sympathetic ganglia electrically stimulated *in vivo.* J. Cell Biol. *60:* 365–374.

Salkoff, L., and L. Kelly (1978) Temperature induced seizure and frequency dependent neuromuscular block in a ts mutant of *Drosophila.* Nature *273:* 156–158.

Smith, D. O. (1988) Statistical evidence for non-random clustering of synaptic vesicles associated with filamentous interconnections. Brain Res. *447:* 145–148.

Teichberg, S., and E. Holtzman (1973) Axonal agranular reticulum and synaptic vesicles in cultured embryonic chick sympathetic neurons. J. Cell Biol. *57:* 88–108.

Torri-Tarelli, F., F. Grohovaz, R. Fesce, and B. Ceccarelli (1985) Temporal coincidence between synaptic vesicle fusion and quantal secretion of acetylcholine. J. Cell Biol. *101:* 1386–1399.

Torri-Tarelli, F., C. Haimann, and B. Ceccarelli (1987) Coated vesicles and pits during enhanced quantal release of acetylcholine at the neuromuscular junction. J. Neurocytol. *16:* 205–214.

Tremblay, J. P., and E. Philippe (1981) Morphological changes in presynaptic terminals of the chick ciliary ganglion after stimulation in vivo. Exp. Brain Res. *43:* 439–446.

Wiley, R. G., C. Spencer, and J. J. Pysh (1987) Time course and frequency dependence of synaptic vesicle depletion and recovery in electrically stimulated sympathetic ganglia. J. Neurocytol. *16:* 359–372.