REVERSIBLE ALTERATIONS IN THE NEUROMUSCULAR JUNCTIONS OF *DROSOPHILA MELANOGASTER* BEARING A TEMPERATURE-SENSITIVE MUTATION, *shibire*

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ABSTRACT

In this study we report a relationship between the ultrastructure of the neuromuscular junctions of tibial muscles and the temperature-induced paralysis in *shibire* flies. There is a decrease in the number of synaptic vesicles of neuromuscular junctions in flies which are held at or above 29°C. Shortly after return to 22°C, the synaptic vesicles are again present in large numbers. Prior treatment with tetrodotoxin or barbiturate protects the junctions from the temperature-induced change in morphology.

KEY WORDS neuromuscular junction · temperature-sensitive · synaptic vesicles · *shibire*^{ts} · mutant

The structure, function, and development of the nervous and muscular systems are strikingly similar across many diverse phyla. Thus, it is reasonable to exploit the particular advantage of one organism, *Drosophila melanogaster*, under the assumption that a genetic approach will facilitate the dissection of complex problems in physiology and development.

The strategy of selecting flies that are paralyzed at a restrictive temperature (12) has yielded a number of mutations at several loci (1). One mutant in particular has been the subject of careful analysis in several laboratories. This mutant, *shibire*^{ts1} (*shi*^{ts1}), is characterized by reversible paralysis of both adult flies and larvae at temperatures above 28°C (1). It also exhibits a number of diverse phenotypic responses to heat pulses administered at different developmental stages (7). A defect in some aspect of neural function was suggested by the reversible loss of on- and off-transients of electroretinograms in *shi*^{ts1} flies at the restrictive temperature (5). The paralysis has been localized by electrophysiological methods to transmission failure at the neuromuscular junction (11, 3), since at high temperature the presynaptic nerve was able to conduct impulses and the muscle responded to direct stimulation. Further examination of this system, where presumably the temperature sensitivity of a single gene product is responsible for the effects on nervous activity, may be useful in understanding both synaptic transmission and the turnover processes of the synaptic vesicles at a molecular and genetic level.

In this study we report a relationship between the ultrastructure of the neuromuscular junction of tibial muscles and the temperature-induced paralysis in *shibire* flies.

MATERIALS AND METHODS

The principal experimental strain used in this study was *shi*^{ts1}. Oregon R served as the wild-type control. One experiment used *shi*^{ts6}/*df(1)sd*^{72a26}, in which *shi*^{ts6} is left

in the hemizygous condition by a small deficiency which includes the locus. 1- to 3-d-old female flies which were grown at 22°C on standard cornmeal, sugar, yeast, and agar medium were used in all studies.

Heat pulses were usually delivered to flies in small shell vials immersed in a water bath. The temperature inside the vial, measured by a YSI thermometer probe, reached water bath temperature within 30 s.

For drug administration, flies were ether-anesthetized and immobilized on scotch tape on the surface of a microscope slide. They were further restrained by a rayon fiber across the neck with its ends anchored on the tape. Flies recovered from the ether within ~10 min. At 30 min the actively struggling flies were injected into the abdominal hemocoel with ~1 μl of tetrodotoxin (1 mg/ml in H_2O or 3 mg/ml phenobarbitol in 10% ethanol. Within a few seconds all movement ceased. The slide carrying the flies was placed in a plastic petri dish submerged in a water bath at 29.5°C for 20 min. For controls, tetrodotoxin-treated flies were kept at 22°C for 20 min before fixation.

The fixation procedure involved rapidly removing a leg from the fly and submerging it in a large drop of fixative. It was cut across the distal femur and then at the distal tibia for rapid fixative penetration. This operation was carried out under a dissecting microscope (Wild Heerbrugg Instruments Inc., Farmingdale, N. Y.). Legs were fixed individually to keep the period from heat pulse to fixation under 20 s. After dissection, the legs were transferred to a vial of fresh fixative for a period of 4 h to overnight. Since the cuticle is hydrophobic and floating precluded fixation, the legs were kept submerged during the initial fixation. The primary fixative used was a cacodylate-buffered formaldehyde-glutaraldehyde solution, one-half the concentration of Karnovsky's fixative (4). Control flies were fixed at room temperature, and experimental flies were fixed at the temperature of the heat pulse. For the very brief heat pulses, animals were placed on the surface of a T75 culture flask filled with water which was siphoned from a heated reservoir. A large drop of fixative was placed beside the group of flies, and both were covered with the top from a 25-mm plastic petri dish. The temperature was recorded from a thermister probe next to the flies and was regulated by the flow of hot water through the T flask. This setup permitted continuous observation of paralysis under a Wild M5 dissection microscope and rapid fixation of the legs at the end of the heat pulse. They were postfixed in 1% osmium tetroxide (6, 14) for 1 h, prestained en bloc with 1% aqueous uranyl acetate solution for 1 h, dehydrated through ethanol, and embedded in Epon-Araldite resin. 60- to 80-nm sections were cut on a Porter-Blum MT-2 microtome (DuPont Instruments, Wilmington, Del.) and stained with 5% aqueous uranyl acetate for 10 min at 60°C followed by Reynolds' (8) lead citrate for 5 min. Grids were examined on a JEOL 100B electron microscope at 60 or 80 kV.

RESULTS

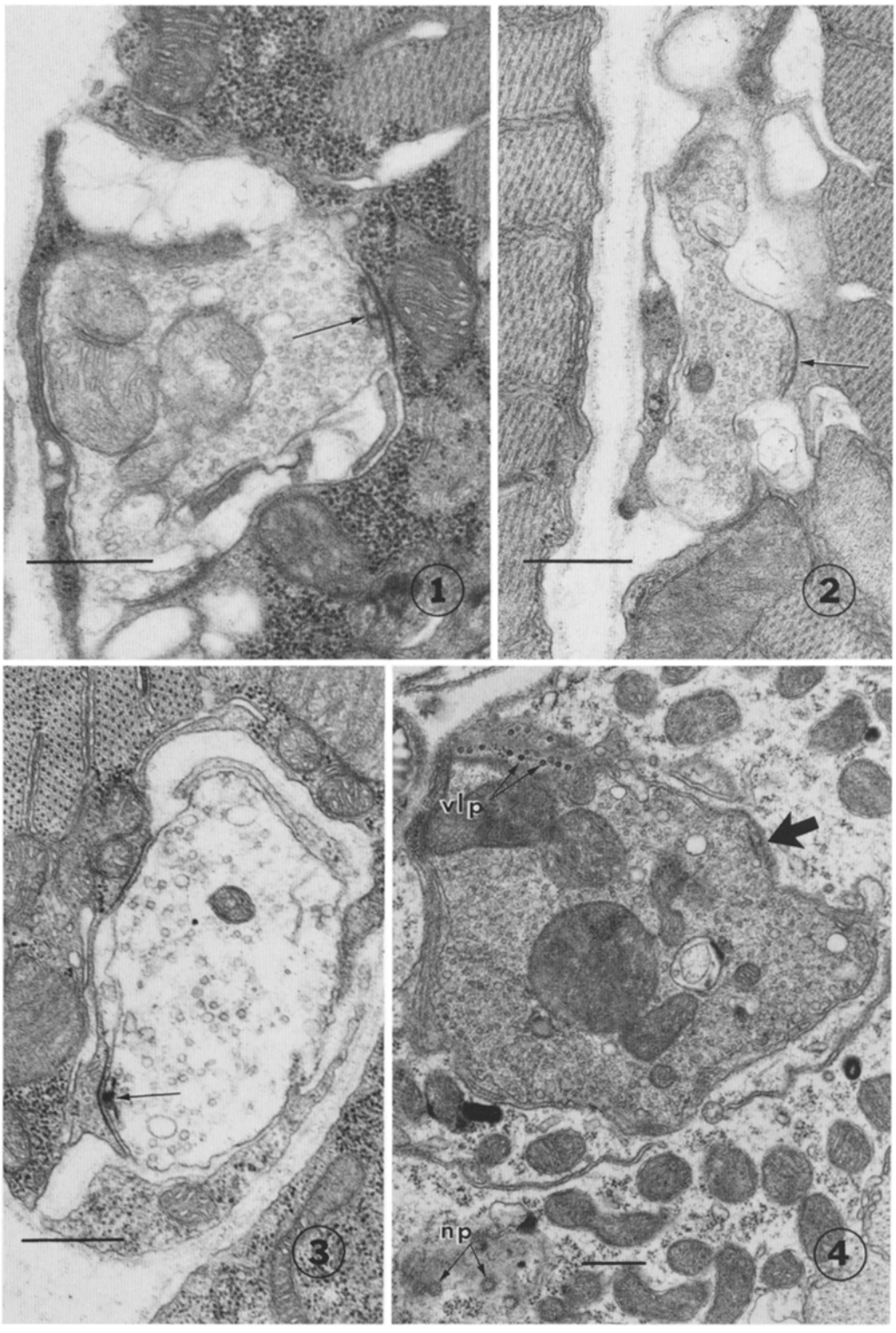
Because the *Drosophila* tibia contains a large number of easily identified neuromuscular junctions, it was chosen for these experiments. Two types of neurons are seen: Large axons with correspondingly large synapses are found at the apices of the oval cross sections of muscle fibers. Smaller axons with smaller synapses lie along the sides of fibers. Both kinds of axon run along the long axis of the muscle fiber, making synaptic contacts at intervals. Thus, in serial cross sections several synaptic regions from the same nerve are found. A diagnostic feature of the neuromuscular junction is the darkly staining synaptic complex, a landmark which is present in both controls and experimental flies. Both types of nerve endings show similar responses to experimental programs. In each experiment, neuromuscular junctions from several flies were examined and from one to as many as 12 separate axons were observed per fly. In most cases where multiple neuromuscular junctions were seen, their morphology was consistent.

Controls

Cross sections of axons at neuromuscular junctions of three Oregon-R flies (one at 22°C, two at 29°C) typically show a large number of synaptic vesicles throughout the axon and clustered around the characteristically dark-staining synaptic complexes (Figs. 1 and 2). A small number (two to three per section) of large cisternae are seen. No appreciable difference is seen in 22° or 29°C specimens. Neuromuscular junctions of 6 *shi*^{ts1} flies held at 22°C are very similar to the Oregon-R neuromusclar junctions, with a high concentration of synaptic vesicles and a small number of cisternae (Fig. 3).

Experimentals

Neuromuscular junctions of flies heat pulsed (thus paralyzed) for from 1.5 min to 3 h were examined. Five flies fixed afer 1.5 min at 29°C (paralysis occurs by 0.5 min) showed little increase in cisternae and no clear change in synaptic morphology (Fig. 4), but in the sample of 10 flies kept at 29°C for 2–3 h there was a striking morphological change compared with the six controls (Figs. 5–7): synaptic vesicles were greatly reduced in number or, in some cases, absent, and the number of large cisternae increased considerably. Four flies held at 29°C for 20 min showed a similar


1
2
3
vlp
np
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neuromuscular junction morphology. Four flies fixed after 2.5 min had few vesicles and extensive cisternae. Neuromuscular junctions of two flies held at 29.5°C for 5 min before fixation showed both an increase in the number of cisternae and vesicle depletion. Likewise, two flies held at 34.4°C for 4 min showed many cisternae and very few vesicles. Neuromuscular junctions from 3 $shi^{ts6}/df(1)sd^{72a26}$ flies after 5 min at 30°C also showed few vesicles and many cisternae; a fourth fly had neither vesicles nor cisternae at the two junctions observed. A group of 30 flies was heated to 29.5°C for 5.25 h, then transferred to the stage of the dissection microscope at 22°C. Most of the flies began kicking in 1.5–3 min and were able to right themselves in 3–5 min. Several that were able to walk by 5 min were fixed within 7 min of their return to 22°C. They all showed large numbers of synaptic vesicles. More cisternae were present than in untreated flies, but not so many as seen in flies fixed immediately after a long heat pulse (Fig. 8).

In summary, the effects of long heat pulses were clear. The neuromuscular junctions were depleted of synaptic vesicles but contained numerous large membranous cisternae. This was the only effect on morphology that was noticed in the nerves and muscles. Other structures such as neurotubules, mitochondria, and muscle filaments appeared similar in controls and experimental flies. Fixation after shorter heat pulses or after a brief recovery period resulted in an intermediate and variable degree of alteration in the morphology of the junction. Heat pulses of 1 min, although long enough to paralyze an animal, resulted in some junctions that were not distinguishable from those of controls.

The administration of tetrodotoxin or phenobarbitol to *shibire* flies causes an immediate irreversible paralysis. However, such a treatment does not have a profound effect on the morphology of the neuromuscular junction (Fig. 9) in the 20 min before fixation. Furthermore, pretreatment with these drugs followed by a 20-min exposure to 29.5°C resulted in neuromuscular junctions which were indistinguishable from those of controls or *shibire* animals given only a brief heat pulse (Figs. 10 and 11). That is, the drugs seem to protect the morphology of the nerve from changes which normally occur in a mutant at the restrictive temperature.

When relating the change in morphology to the temperature-sensitive mutant *shibire*, it is important to rule out the possibility that a different temperature-sensitive mutation coincidentally present in this strain might be involved. Since independent mapping of the paralysis effect and the synaptic vesicle effect by recombination would be impractical, we have relied on deficiency and duplication mapping. When shi^{ts6} is left hemizygous by a small deficiency ($df(1)sd^{76a26}$) which includes the locus, both paralysis and the loss of vesicles occur at the restrictive temperature, as noted earlier. Conversely, a small duplication of shi^{+} translocated to the Y chromosome protects the fly indefinitely at 29°C. While the ultrastructure of this strain has not yet been examined, we think it unreasonable to expect a loss of synaptic vesicles since paralysis does not occur. The deficiency limits any putative second mutation to 13F1-14B1 on the salivary chromosome map. The duplication sets similar narrow limits.

DISCUSSION

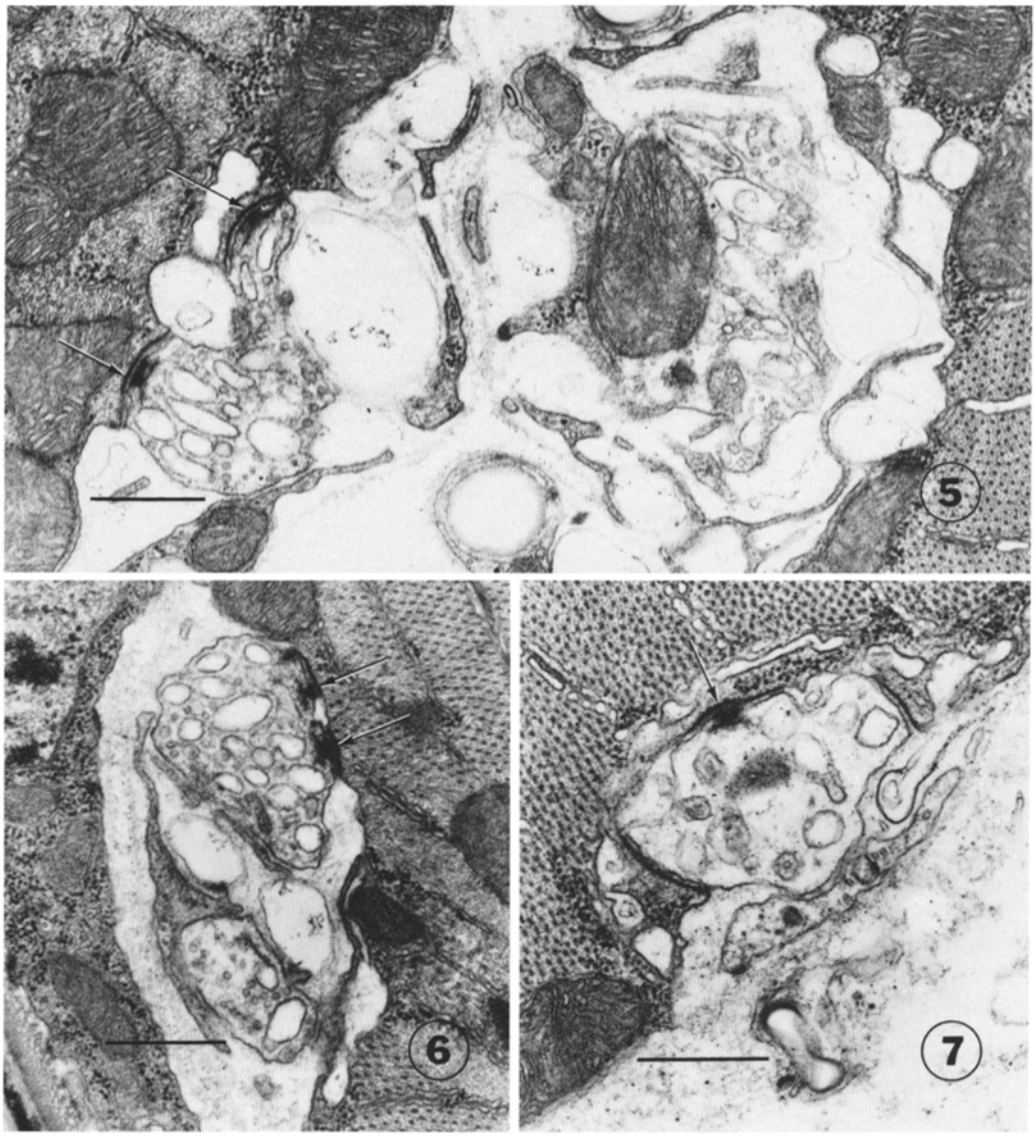
We have found that there is a clear morphological effect of temperature on the neuromuscular synapse of $shibire^{ts}$ flies. Synaptic vesicles disappear within 2.5 min after the onset of paralysis, and

FIGURE 1 Neuromuscular junction in tibia of Oregon-R fly maintained at 22°C. Arrow points out synaptic complex. Bar, 0.5 μm. × 39,000.

FIGURE 2 Neuromuscular junction in tibia of Oregon R fly held at 29°C for 2 h. Arrow points out synaptic complex. Bar, 0.5 μm. × 33,000.

FIGURE 3 Neuromuscular junction in tibia of shi^{ts1} maintained at 22°C. Arrow points out synaptic complex. Bar, 0.5 μm. × 31,000.

FIGURE 4 Neuromuscular junction in tibia of shi^{ts1} fly held at 29°C for 90 s. Arrow points out synaptic complex. *np*, nuclear pores; *vlp*, unidentified viruslike particles. Note abundant synaptic vesicles. Bar, 0.5 μm. × 19,600.



FIGURES 5–7 Neuromuscular junctions in tibias of *shi*^{ts1} flies held at 29°C for 2 h. Arrows point out synaptic complexes. Note presence of large vesicles or cisternae and near absence of synaptic vesicles. Bar, 0.5 μm. Figs. 5 and 6, × 31,000. Fig. 7, × 37,000.

reappear within a few min after return to the permissive temperature. This reduction in the number of vesicles is accompanied by the appearance of large cisternae.

The time-course of vesicle depletion suggests that these morphological changes are not the primary effect of high temperature on *shibire*, nor the immediate cause of paralysis. This is suggested by the healthy appearance of synapses in flies heat pulsed for 90 s and thus paralyzed for 60 s before fixation. In addition, the experiments involving treatment with tetrodotoxin or phenobarbitol before heat pulsing reveal the apparent need for nerve activity to deplete synaptic vesicles.

Direct observation of flies experiencing a heat pulse reveals that activity accelerates as the temperature approaches 29°C. The flies lose coordination rapidly but vigorous twitching of their legs,

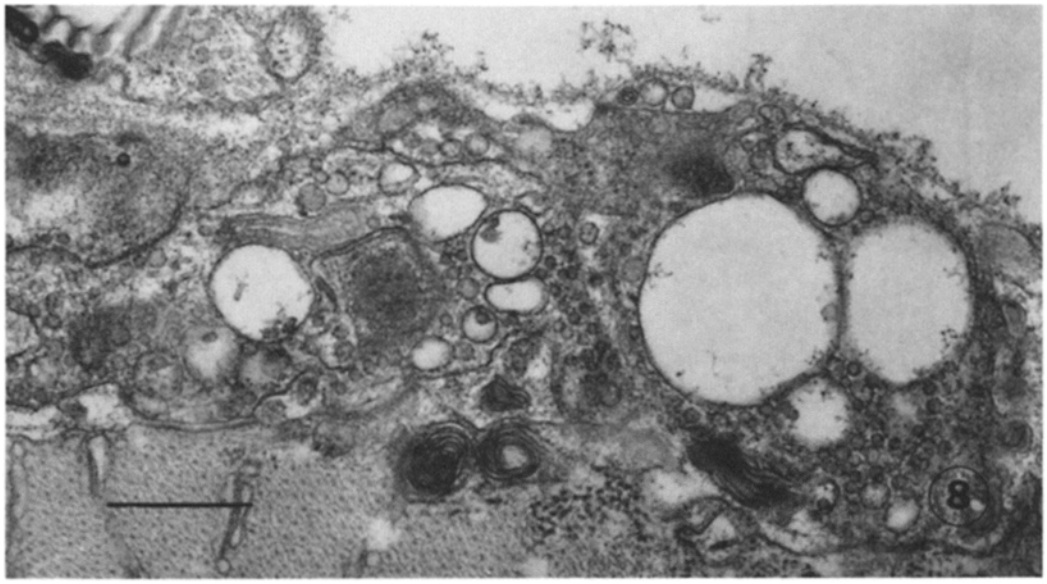


FIGURE 8 Neuromuscular junction in tibia of *shi*^{ts1} fly paralyzed at 29°C for 5½ h and allowed to recover for 7 min. Note presence of both synaptic vesicles and cisternae. Bar, 0.5 μm. × 40,000.

wing flicking, and buzzing precede a gradual slowdown of activity over a 20-s period until all movement ceases except for the occasional twitch. The appearance is that of catatonic rather than flaccid paralysis and is what one might expect from an initially hyperactive nervous system followed by exhaustion.

Salkoff and Kelley (10) have found that the muscular paralysis in *shi*^{ts1} is preceded by muscle firing due to spontaneous ganglionic activity. The ganglionic firing can be prevented by excision of the ganglion or by perfusion with barbiturates, and also does not occur at the low end (28°–28.5°C) of the range of temperature in which paralysis occurs. In these cases, the ganglion-induced muscle firing does not occur, and the neuromuscular synapse will continue to transmit in response to applied stimulation. Under these conditions without ganglion input, transmission at the neuromuscular synapses in the mutant nevertheless fails at frequencies of stimulation >0.6 Hz. However, there is a rapid recovery of the ability to transmit when external stimulation is stopped.

The appearance of an increase in the number of cisternae and depletion of synaptic vesicles after 2 min at high temperature is very similar to the appearance of the frog neuromuscular junction stimulated at 10 Hz for 15 min (2) or depolarized by high levels of extracellular K^+ for 30 min (9). The similarity in ultrastructure in the two organisms is consistant with the notion that one effect of high temperature on *shibire* is a depolarization of the presynaptic terminal. However, such an effect was not noticed by Ikeda et al. (3) who applied stimuli to the mesothoracic leg nerve and recorded muscular response in *shi*^{ts1} at 19°C and its absence at 29°C. They found that excitability and conduction were not affected by the temperature changes, a conclusion also reached by Siddiqi and Benzer (11). Since a train of action potentials occurred in muscle membrane in response to direct muscle stimulation, a block at the synaptic junction was inferred (11). Thus, a reasonable explanation for the initial *shibire* defect, the block in neuromuscular transmission and paralysis, postulates a postsynaptic block, a defect of either the receptor itself, or of the membrane in which it sits. Since any hypothesis on the nature of the *shibire* defect and the mechanism of paralysis would also have to account for the pleiotropic effects of the mutation on a wide variety of cell types in the developing animal, some of which seem membrane associated (7, 13), a general membrane defect seems the more likely explanation.

Under the hypothesis of a postsynaptic block, depletion of the presynaptic terminal could be explained by the ganglionic firing, although how

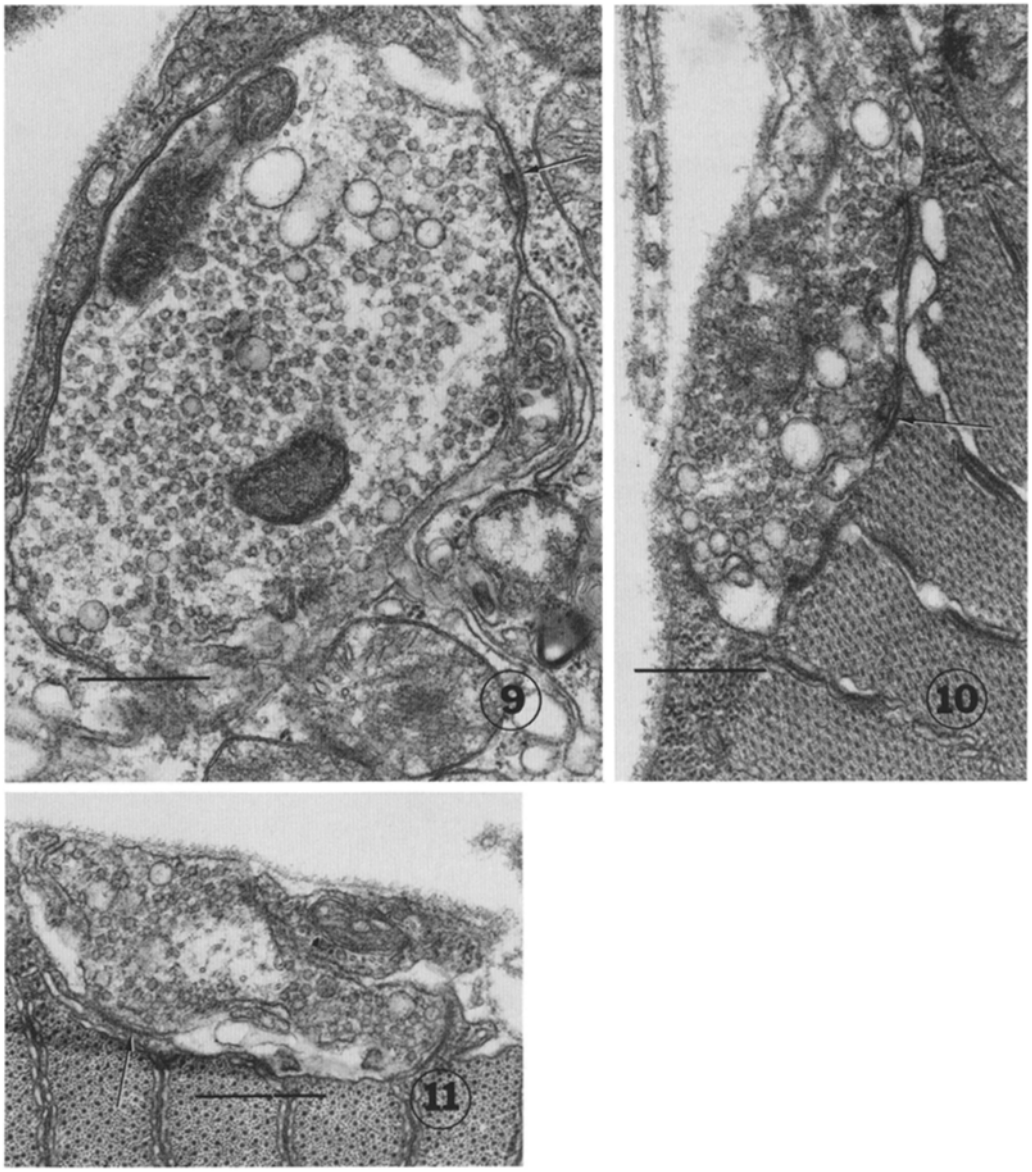


FIGURE 9 Neuromuscular junction in tibia of *shi*^{ts1} fly injected with tetrodotoxin and held for 1/2 h at 22°C before fixation. Arrow points out synaptic complex. Bar, 0.5 μm. × 34,000.

FIGURE 10 Neuromuscular junction in tibia of *shi*^{ts1} fly that had been injected with tetrodotoxin and held for 1/2 h at 29°C before fixation. Arrow points out synaptic complex. Bar, 0.5 μm. × 34,000.

FIGURE 11 Neuromuscular junction in tibia of *shi*^{ts1} fly that had been injected with phenobarbitol and held for 1/2 h at 29°C before fixation. Note abundant vesicles and a moderate number of larger vesicles or cisternae. Arrow points out synaptic complex. Bar, 0.5 μm. × 34,000.

this firing is caused by the same *shibire* defect occurring more centrally in the nervous system is as yet unexplained. Prolonged or repeated depolarization at the neuromuscular junction could possibly result in the morphological changes described in this paper. However, wild-type *Drosophila* can tolerate a stimulus frequency of 25 Hz without a decline in neuromuscular transmission

(10), and the rate of spontaneous firing from the ganglion reported was 3–6 Hz, so that it is questionable whether this stimulus is sufficient to cause the depolarization seen. An alternative explanation for the depleted terminals is that a block or bottleneck in the process of forming or recycling synaptic vesicle material also occurs. Such an effect could not be the initial cause of paralysis but could account for the morphology of the junction after a prolonged heat pulse. Under this interpretation, the paralysis and depletion of synaptic vesicles would be independent effects of the initial *shi* defect.

In summary, a heat pulse of 29°C causes rapid paralysis of flies bearing the mutation *shibire*^{ts1} and also effects a dramatic loss of synaptic vesicles over a longer time period. Resolution of the relationship between the paralysis and the change in morphology of the nerve terminals requires a more extensive analysis of the physiology of the nerves both intact and removed from the control of the ganglion. Further characterization of the temperature-sensitive gene product of *shibire* and its involvement with the nerve membrane in such a readily reversible system may be useful in investigating synaptic transmission and may provide insight into how the transmitter is packaged and membranes are recycled.

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