Reversible Control of Synaptic Transmission in a Single Gene Mutant of *Drosophila melanogaster*

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ABSTRACT Synaptic transmission of the single gene mutant, *shibire*^{ts1} (*shi*), of *Drosophila melanogaster* is reversibly blocked by elevated temperature. The presynaptic mechanism of transmission was studied in the neuromuscular junction of the dorsal longitudinal flight muscle of this mutant. It was observed that when the temperature was raised to 29°C in *shi* flies, the amplitude of the excitatory junction potential (EJP) greatly diminished, the frequency of spontaneously released miniature excitatory junction potentials (MEJP's) was greatly reduced, and almost complete vesicle depletion was observed. These conditions were reversible if the temperature was lowered to 19°C. These data suggest that the block in transmission is a result of vesicle depletion. It is suggested that depletion occurs not as a result of excessive release of transmitter but rather as a result of a block in the recycling of vesicles, which causes depletion as exocytosis (transmitter release) proceeds normally.

Since the discovery of the quantal nature of transmitter release (2), the release mechanism has been investigated physiologically at various synapses, resulting in a general acceptance that the quantal nature of transmitter release can be generalized to various synapses in a variety of animals. This was followed by the morphological finding of synaptic vesicles (3, 20), and the presence of such vesicles at the terminal has also been shown to be generalizable to various types of chemical synapses.

The localization of vesicles of discrete size at the presynaptic terminal suggested a possible mechanism for the release of transmitter molecules in discrete packets. Thus, an effort has been made to investigate the relationship between these physiological and morphological findings. A parallelism between intensive synaptic activity and the depletion of vesicles has been shown, for instance, by the use of black widow spider venom (1, 6, 22, 27). The irreversible nature of the venom action, however, makes the interpretation of the results difficult. Other methods of inducing transmitter release, such as prolonged stimulation (7, 29), application of 4-aminopyridine (8, 26), and increasing external potassium (5), have also resulted in vesicle depletion. These works are consistent with the hypothesis that transmitter is released through the exocytosis of vesicles. On the other hand, less biologically severe methods of inducing transmitter release have failed to produce an effect on the number of vesicles (for review, see reference 14). Furthermore, transmitter release during synaptic activity has been claimed to be of cytoplasmic origin rather than vesicular (4, 17). Thus, the relationship between the release of transmitter and synaptic vesicles is at present unclear.

To investigate the mechanism of transmitter release in relation to synaptic vesicles, we studied the neuromuscular junction of a mutant *Drosophila*, *shibire*^{ts1} (*shi*), in which the release of transmitter is controllable by changing the temperature. The amplitude of the neuromuscular excitatory junction potential (EJP) of this mutant progressively diminishes as the temperature is raised >27°C, leading to an almost complete disappearance of the potential at 30°C, while the function of the postsynaptic membrane and contractility of the muscle fiber remain normal (10, 25).

In addition, depletion of vesicles at the neuromuscular junction following temperature-induced motor activity has also been reported in this mutant (11, 21). However, no study has been done using both electrophysiological and morphological techniques to correlate the disappearance of the EJP with the loss of vesicles at the synapse. This is the purpose of the present paper. We report a parallelism between the loss of the EJP, loss of miniature excitatory junction potentials (MEJPs), and loss of synaptic vesicles.

MATERIALS AND METHODS

The neuromuscular junction of the dorsal longitudinal flight muscle (DLM) of *Drosophila melanogaster* was used in this study. The DLM is composed of six fibers which attach anteriorly to the dorsal thoracic cuticle and posteriorly to the posterior phragma. Each fiber is multiterminally innervated by a single excitatory motor neuron (12). The DLM motor neurons send their axons through the posterior dorsal mesothoracic nerve (PDMN).

Adult female flies (3–4 d after eclosion), homozygous for the *shi* gene, were used for the experiment, and adult wild-type females (Oregon-R) were used as the control. Both the mutant and wild-type flies were reared at 17 ± 1°C and handled during the experiment in a low-temperature room (19 ± 1°C).

For the physiological experiments, the fly was mounted with Tackiwax laterally over an opening in a plastic tube, so that its underside remained exposed to air, while the preparation was 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) at 19°C. The mesothoracic pre-episternum, pleurum, and the dorsoventral muscle 1 (DVM 1) were removed, exposing the lateral surface of the mesothoracic ganglion, the PDMN innervating the DLM, and the lateral surface of the DLM. The PDMN was cut at a point ~20 µm from the thoracic ganglion. The cut end was sucked into a glass capillary electrode containing saline solution for stimulation.

For the evoked response, the PDMN was stimulated with a 0.1 ms square pulse at a rate of 0.5 Hz. Recordings of the evoked response were made using any of the six fibers, while recordings of the MEJPs were made using muscle fibers 5 or 6 (18), the two smallest fibers of the DLM. The resting potentials of the muscle fibers were checked at the beginning and end of each physiological experiment, and only preparations which showed constant potentials of −90 mV or more were used.

Postsynaptic potentials were recorded via an intracellular electrode inserted into the muscle fiber at its lateral surface or through the cuticle at the tergal end of a muscle fiber. The electrical event was monitored differentially through a set of high-input impedance DC amplifiers and displayed on an oscilloscope and photographed. Both the intracellular and reference electrodes were filled with 2.5 M potassium acetate and connected to the amplifier via Ag-AgCl wires. The temperature was controlled by a thermoelectric unit placed under the plastic dish and monitored by a thermister placed in the saline solution ~1 mm from the fly. The temperature was changed at a rate of ~1°C/min.

The evoked responses of 18 wild-type flies and 23 *shi* flies were observed as the temperature was raised and lowered between 19 and 30°C. The MEJPs of eight wild-type flies and 13 *shi* flies were observed as the temperature was raised and lowered over the same range.

For electron microscopic observation, the fly was dissected as described above, and the temperature of the saline was then set at the desired experimental level. After exposure to the experimental temperature for 5 min, the fly was immediately immersed in a fixative kept at that temperature. As a test for possible artifacts derived from treatment of the fly in the saline solution before fixation, some flies were immersed in fixative immediately after quick hemi-section along the fly's midline. In either case, the fly was fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 20–30 min at the experimental temperature. It was then further fixed in 4% glutaraldehyde in the same buffer solution for 1.5–2 h at room temperature. After postfixation in 2% osmium tetroxide in the same buffer for 2 h at 4°C, the fly was dehydrated with increasing concentrations of ethyl alcohol and embedded in Epon 812. Some flies were stained en bloc with 2% aqueous uranyl acetate for 20 min prior to dehydration. Sections were stained with 2% uranyl acetate in 70% ethyl alcohol and Millonig's lead solution (19) and observed in a Hitachi HU-11B or Philips 301 electron microscope. At 19°C, 25 wild-type flies and 6 *shi* flies were observed. At 30°C, 6 wild-type flies and 6 *shi* flies were observed. For the recovery test, five flies which had been exposed to 30°C for 15 min, then cooled to 19°C at a rate of 1°C/min were observed. An average of about 50 synapses per fly was observed.

RESULTS

Fine Structure

As described previously, the axonal branches of the innervating neuron enter the deep invaginations of the muscle fiber membrane and make multiple synaptic contacts (12, 24). No appreciable changes were observed in the structure of the axon or muscle fiber at temperatures between 19 and 29°C in either *shi* or wild-type flies. Thus, this study focuses on the fine structure of the DLM neuromuscular junction of both *shi* and wild-type flies at various temperatures.

A typical junction of a wild-type fly at 19°C is shown in Fig. 1. The wild-type junction was characterized by the presence of a presynaptic dense body in the presynaptic cytoplasm adjacent to the presynaptic membrane. Many synaptic vesicles (45–50 nm in diameter) were accumulated in the cytoplasm around the presynaptic dense body. The presence of mitochondria, neurotubules, and a few vacuoles was also noted. The pre- and postsynaptic membranes were separated by a space 20–25 nm in width. The postsynaptic membrane was accompanied by granular substances on the synaptic cleft side and a fine, filamentous undercoating on the cytoplasmic side. In the postsynaptic cytoplasm, bundles of myofibrils, large mitochondria, and glycogen granules were observed. The structure of the junctions of *shi* flies at 19°C (Fig. 2) was indistinguishable from that of the wild-type.

At 29°C, the structure of the wild-type synapse remained the same as at 19°C (Fig. 3), whereas remarkable changes were observed in the *shi* synapse (Fig. 4). In the presynaptic terminals of *shi* at 29°C, almost no vesicles, but many cisterna-like structures, were observed. The other structures, such as the presynaptic dense body, the granular substances, and the filamentous undercoating at the postsynaptic membrane remained unchanged. In some cases, infolding of the presynaptic membrane near the synapse (Fig. 5) were observed. On the cytoplasmic side of the cisternal membrane, as well as on the infolding, a bristlelike coat was also observed.

To observe recovery, we exposed the *shi* flies to a temperature of 29°C for 5 min. The temperature was then reduced at a rate of 1°C/min to 19°C, 15 min after which the fixation was made. As shown in Fig. 6, the terminals of *shi* contain many vesicles but no cisterna-like structures. The other structures also appeared to be similar to those at low temperature. Thus, the alteration in the structure of the presynaptic terminal of *shi* flies at 29°C was reversible.

Evoked Response

Responses of a DLM fiber to a single stimulation at the PDMN in wild-type and *shi* flies at different temperatures are shown in Fig. 7. The muscle fiber of the wild-type (control) fly always responded with an all-or-none action potential of ~110 mV in amplitude at any temperature between 19° and 30°C. The action potentials of DLM fibers in *shi* flies, at temperatures

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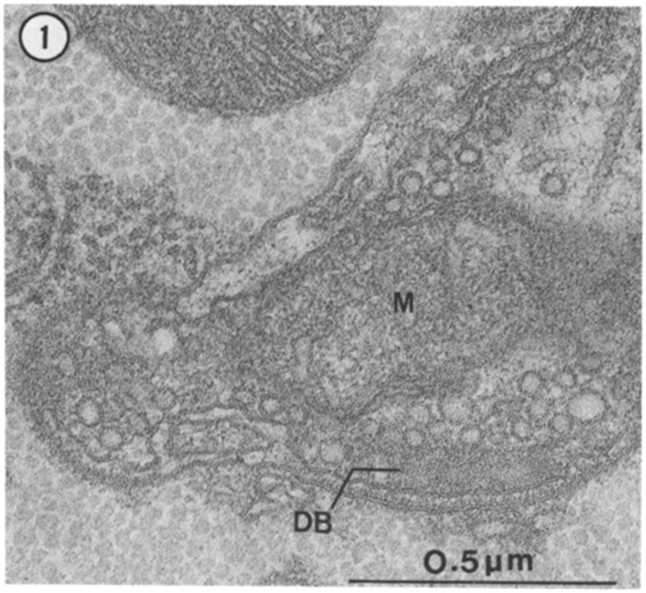
FIGURES 1–6 Fig. 1: Neuromuscular junctions of the DLM from a wild-type fly (Oregon-R) at 19°C. Many synaptic vesicles can be seen around the presynaptic dense body (*DB*). *M*, mitochondrion. Bar, 0.5 µm. × 70,000. Fig. 2: Neuromuscular junctions of the DLM from *shibire*^{ts1} (*shi*) flies at 19°C. *DB*, presynaptic dense body. *Gly*, glycogen granules. Bar, 0.5 µm. × 38,000. Fig. 3: Neuromuscular junction of the DLM from a wild-type fly (Oregon-R) at 29°C. Typical junction at this temperature shows no specific difference from the one at 19°C. Bar, 0.5 µm. × 64,000. Fig. 4: Neuromuscular junction of the DLM from a *shi* fly at 29°C. Typical junction at this temperature shows almost no vesicles but many cisterna-like membranous structures in the terminal. Pre- and postsynaptic membrane, presynaptic dense body, and mitochondria in the terminal remain as those at 19°C. *Sc*, sheath cell. Bar, 1 µm. × 28,000. Fig. 5: Neuromuscular junction of the DLM from a *shi* fly at 29°C. A membrane infolding similar to the cisternal structure is seen (arrow). *DB*, presynaptic dense body. *M*, mitochondrion. Bar, 0.5 µm. × 60,000. Fig. 6: Neuromuscular junctions of the DLM from a *shi* fly after recovery from exposure to 29°C for 5 min. The temperature was raised to 29°C and held at that temperature for 5 min; thereafter, the temperature was lowered to 19°C and held for 5 min before fixation. Synaptic structure appears to be similar to the ones in the control condition. *M*, mitochondrion. *MT*, microtubules. Bar, 0.5 µm. × 44,000.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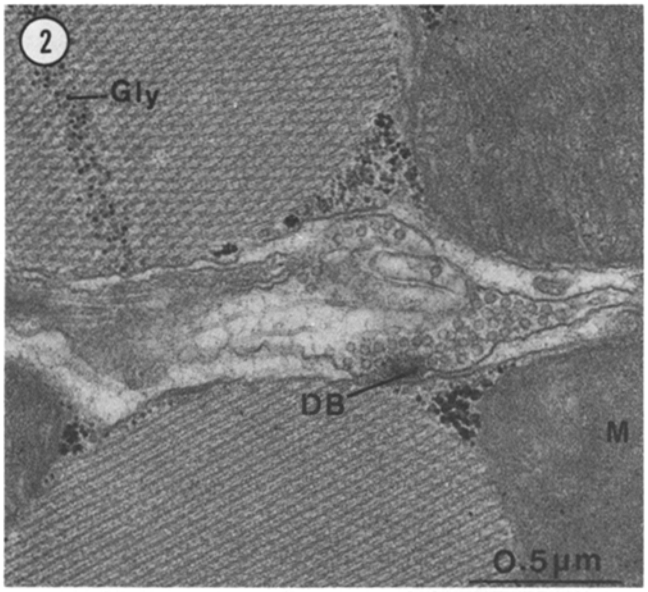
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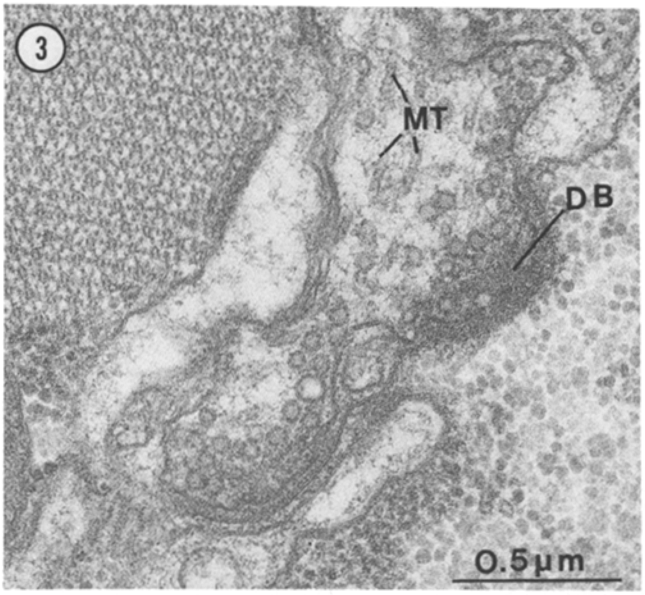
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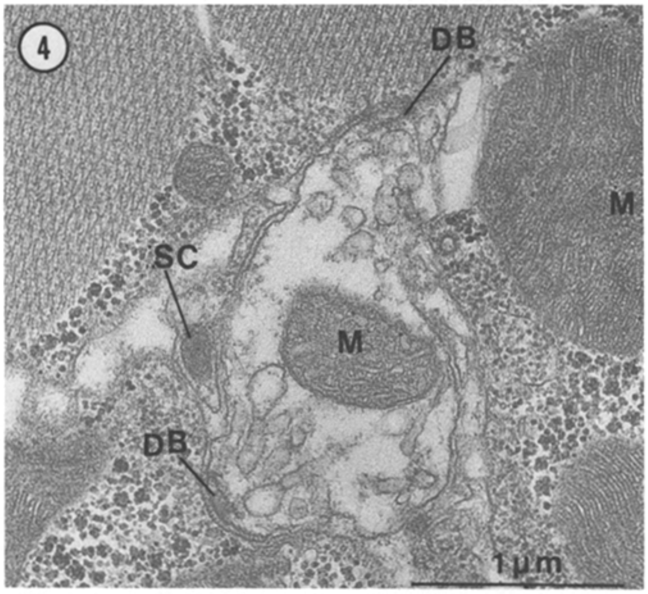
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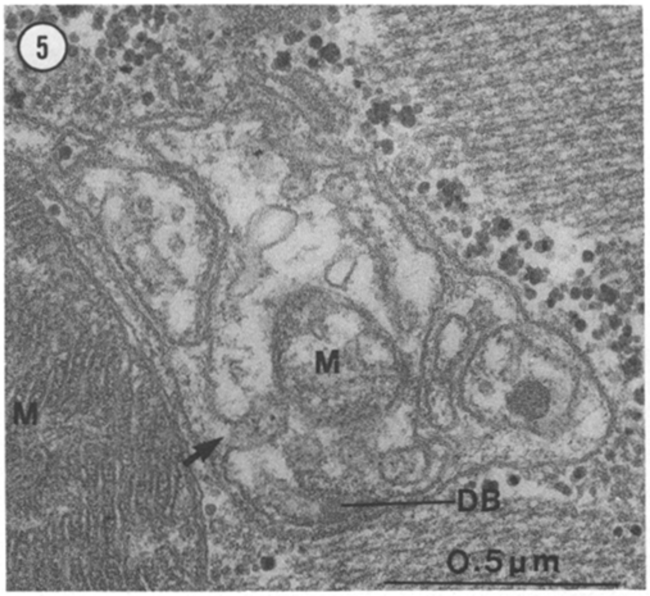
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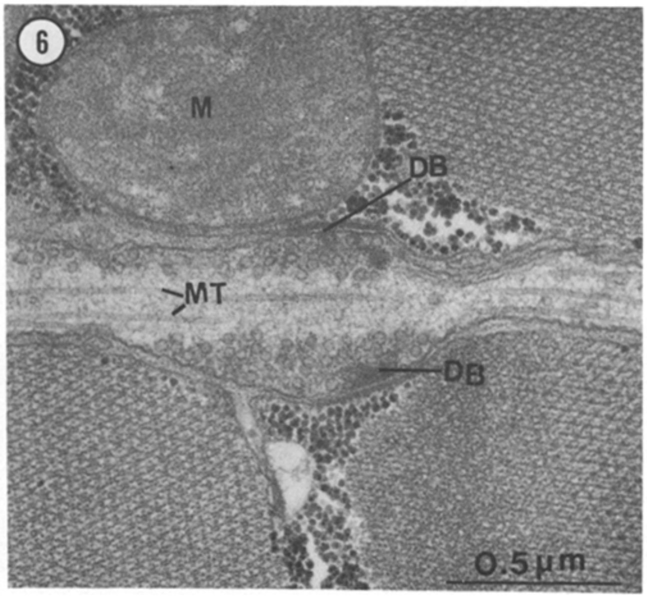
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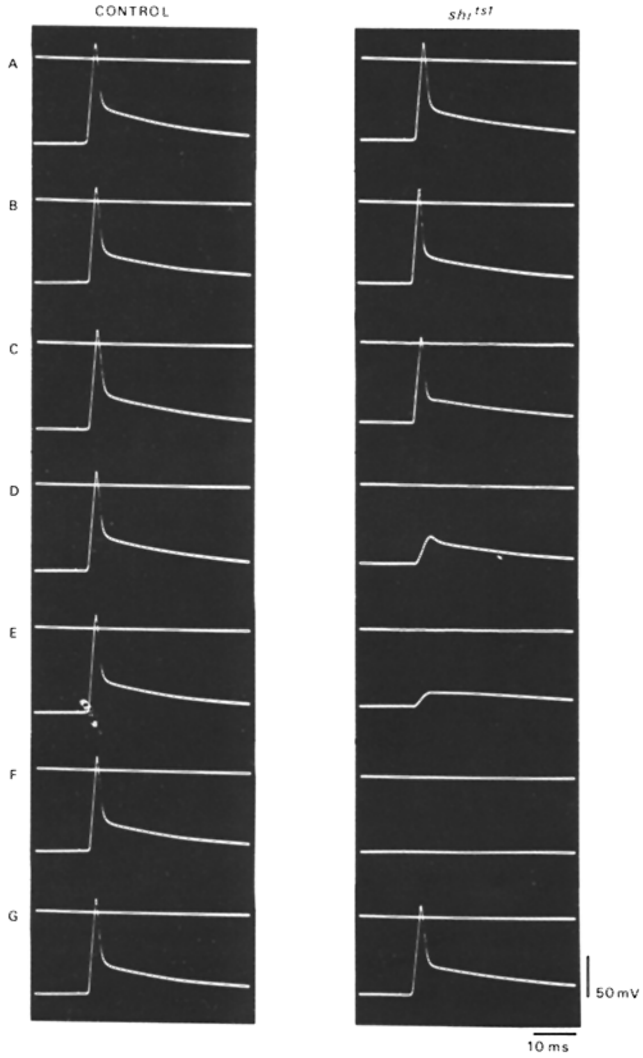

FIGURE 7 Evoked responses of the DLM upon stimulation of the PDMN at various temperatures. Intracellular recordings from the DLM fiber 3 in both control (Oregon-R) and the mutant, *shi*. In each recording, the top trace shows the reference extracellular potential level. The lower trace shows the intracellular potential against the reference level. A small notch on the lower trace ~2 ms preceding each response is the stimulus artefact. (*A*) At 19°C. Both control and *shi* show all-or-none action potentials followed by the slowly decaying falling phase of the junction potential. (*B*) At 26°C. No marked difference is seen either in the control or in *shi*. (*C*) At 27°C. The action potential of *shi* becomes graded. (*D*) At 28°C. The response of *shi* shows only a small regenerative component on top of the junction potential. (*E*) At 29°C. The response of *shi* becomes a junction potential of reduced size only, with no regenerative potential. (*F*) At 30°C. The transmission in *shi* is almost completely blocked. No observable junction potential. (*G*) At 19°C. Recovery from *F*. Calibrations refer to *A* to *G*. 50 mV, 10 ms.

between 19° and 26°C, were indistinguishable from those in the wild-type flies. However, at temperatures between 27°C and 28°C, the action potential of *shi* became graded; and at 29°C the electrogenic component was completely abolished, revealing the diminished neuromuscular junction potential. The amplitude of the junction potential diminished further as the temperature was raised, and disappeared almost completely (under 1 mV) at 30°C. When the temperature was decreased from this level, the amplitude of the junction potential recovered following the decrease in temperature with some hysteresis. At 19°C, complete recovery of the action potential was observed.

Miniature Excitatory Junction Potentials

Typical MEJPs from a fiber of a wild-type fly at 19°C are shown in Fig. 8*A*. MEJPs of various amplitudes were observed, the largest being ~2 mV and the smallest ~50 µV. As the temperature was raised, the frequency of the MEJPs progressively increased until individual events could no longer be distinguished (Fig. 8*B* and *C*). The summating MEJPs gave the impression of an increased noise level, but in both Fig. 8*B* and *C*, the noise level remained the same as that in Fig. 8*A*. When the temperature was decreased to 19°C, the frequency of the MEJPs returned to a level similar to that shown in Fig. 8*A*.

Typical MEJPs from a *shi* fly at 19°C are shown in Fig. 8*D*. As the temperature was raised, the frequency of the spontaneously released MEJPs increased in a similar way to that observed in the wild-type fly (Fig. 8*E*). At 26°C, the temperature-induced central activity (15, 23) caused the motor neuron innervating the muscle fiber to begin firing, resulting in full-sized action potentials in the muscle fiber. As the temperature was raised further, the junction potential diminished gradually until it was only a few mV in amplitude at 29°C (Fig. 8*F*, arrows). MEJPs were observed to decrease in frequency as the temperature was raised and become almost completely absent at 29°C (Fig. 8*F*). Thus, at 29°C, the frequency of the MEJPs was greatly reduced from what normally occurs in a wild-type fly at that temperature (Fig. 8*C*). As the temperature was lowered to 25°C the frequency of the MEJPs began to increase, returning to a frequency typical for that temperature (Fig. 8*G*).

DISCUSSION

The results show a parallelism between the diminution of the EJP, a reduction in the frequency of spontaneously released MEJPs, and synaptic vesicle depletion at the DLM neuromuscular junction of *shi* flies at 29°C. These observations suggest that the reduction in EJP amplitude and the reduction in MEJP frequency may be the result of a reduction in the number of synaptic vesicles available in the presynaptic terminals of the DLM motor neurons.

Vesicle depletion has previously been observed in the neuromuscular junctions of tibial muscles in *shi* flies exposed to high temperature by Poodry and Edgar (21). However, these authors concluded that the observed depletion was not the cause of the neuromuscular transmission block, since they observed that paralysis occurred within 30 s at 29°C, while depletion of vesicles required ~2.5 min at this temperature. This conclusion was based on the assumption that the observed paralysis (cessation of movement) was due to a transmission block at the neuromuscular junction level of the neural pathway controlling leg movement. However, observations by Kosaka and Ikeda (16) show that many different types of synapses in the thoracic ganglion are affected by the *shi* gene, some at temperatures below 29°C. Thus, it is very possible that the leg paralysis observed by Poodry and Edgar (21) was the result of a transmission block, not at the neuromuscular junction level, but at some other level more proximal in the pathway. This interpretation is strenthened by the observation by Poodry and Edgar (21) that during paralysis occasional twitching of the leg occurred. This suggests that transmission at the tibial neuromuscular junction was not blocked at the time of paralysis.

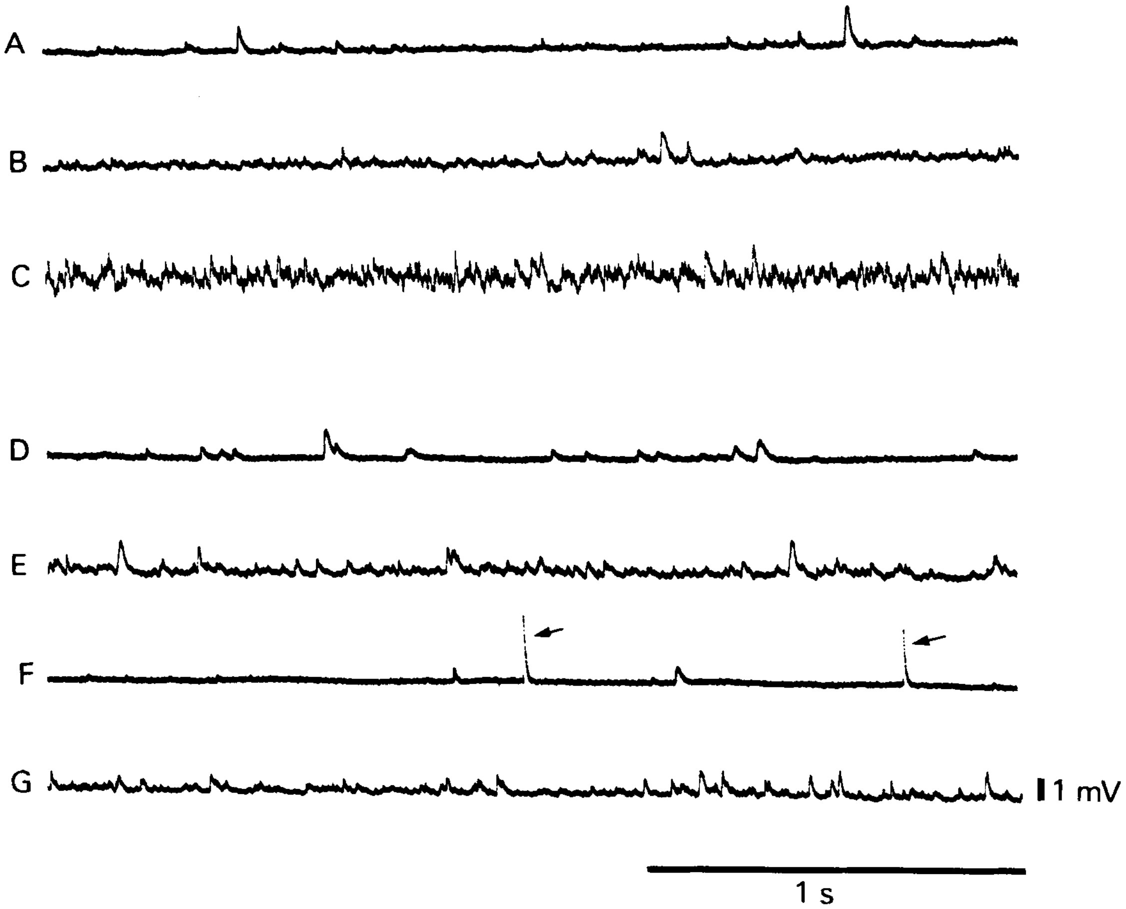

FIGURE 8 Miniature excitatory junction potentials of DLM fiber 6. Wild-type fly at 19°C (*A*), 26°C (*B*), and 29°C (*C*). *Shi* fly at 19°C (*D*), 26°C (*E*), 29°C (*F*). The arrows in *F* point to the greatly diminished EJP. (*G*) Recovery of MEJPs after having been exposed to 29°C for 3 min and then brought to 25°C for 3 min.

Therefore, Poodry and Edgar's (21) observations are not incompatible with the suggestion that the transmission block observed in *shi* flies at 29°C is due to vesicle depletion.

The depleted synapses at 29°C observed in this paper, as well as those reported by Poodry and Edgar (21), are reminiscent of synapses stimulated at high frequency (7, 29) or depolarized by increasing external potassium (5), treatments designed to cause massive transmitter release. However, since the muscle fiber was monitored continuously while the temperature was raised, it was observed that no unusually large amount of transmitter release occurred which would normally result in depletion of these synapses. The centrally induced motor activity was observed to be ~3–6 Hz, which is far below that which causes fatigue in this muscle (25 Hz) (23). Thus, the observed depletion appears not to be due to an excessive release of transmitter.

If excessive release is not the cause of depletion in these synapses, what might be responsible? A clue comes from observations of the *shi* effect on the garland cells, which are involved in uptake of waste products by endocytosis via coated vesicles (28). In the garland cells of *shi* flies at 29°C, an increase in coated pits and a decrease in coated vesicles was observed. Also, HRP uptake was greatly decreased (T. Kosaka and K. Ikeda, manuscript submitted for publication). The results suggested that the endocytotic activity of these cells is blocked at high temperature in *shi* flies, presumably at the step which converts coated pits to coated vesicles.

If this block in endocytosis occurs in nervous tissue as well, then the depletion observed in *shi* could be a result of a blockage in the recycling of synaptic vesicles, which would gradually cause depletion as exocytosis (transmitter release) proceeded normally. Observations by Kosaka and Ikeda (16) on various types of synapses in the CNS of *shi* flies exposed to 29°C suggest that this may be the case. Thus, in various synapses, many pitlike structures were observed lined up along the presynaptic membrane, suggesting an arrested step in vesicle formation. (Such structures were also observed in the DLM synapses, although rarely [T. Kosaka and K. Ikeda, observations to be published separately].)

The published observations on the effect of *shi* all fit well with the possibility that the *shi* gene is affecting the recycling of synaptic vesicles. For example, if the recycling process were blocked when the temperature was raised, then a gradual reduction in the EJP amplitude and a reduction in the frequency of MEJPs would be expected as exocytosis proceeded uninhibited. This is what we report here. Furthermore, if exocytosis (transmitter release) were blocked while the temperature was raised, no depletion would occur and a normally sized response should occur at 29°C. This was observed by Salkoff and Kelly (23), who blocked the centrally induced activity with barbiturates and observed that a normally sized response occurred at 29°C when the motor neurons were stimulated. Furthermore, Poodry and Edgar (21) observed that depletion did not occur in the tibial neuromuscular junctions if tetrodotoxin or barbiturates were applied prior to elevating the temperature. Thus, both depletion and reduction of the

EJP amplitude appear to be dependent on activity (exocytosis).

The possibility that the *shi* gene might be having a postsynaptic effect has been suggested, primarily to explain how the central activity which is also induced by temperature in *shi* might occur (21, 23). However, this activity could easily be explained as being caused by disinhibition of the neuron(s) driving the motor output pattern, which would occur if transmission were blocked in the inhibiting neuron(s). It is not unlikely that the flight system is under inhibition, since application of picrotoxin, an antagonist of the inhibitory transmitter, gamma-aminobutyric acid, to the thoracic ganglion of wild-type flies also causes motor output to the DLM (9). Furthermore, a recent analysis of bilateral mosaic flies showed that the transmission block of the DLM in *shi* correlates with a mutant motor neuron (presynaptic), but not a mutant muscle fiber (postsynaptic), which implies that the transmission block is due to a defect of the presynaptic cell (13). Thus, all the observations that have been made on *shi* are compatible with the possibility that a block in recycling of synaptic vesicles is responsible for the observed vesicle depletion, which is in turn responsible for the block in transmission. Furthermore, our observations are compatible with the hypothesis that, in this system, transmitter is released through the exocytosis of vesicles.

The ability to reversibly manipulate the synaptic transmission mechanism by such a biologically benign method as raising the temperature offers a unique and powerful technique for the study of transmission. Many of our observations suggest that this mutant will provide a useful tool for unraveling the various steps involved in the transmission mechanism. For example, the appearance of large cisternae in the vesicle-depleted terminal suggests a possible relationship between the vesicular membrane and cisternae in the recycling mechanism. Also, the effect of nervous activity on evoked and spontaneous release will perhaps elucidate the relationship between these two phenomena. However, until the quantitative analysis which is in progress is completed, we will refrain from further discussion of the implications of these observations.

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