Relationship of the Reserve Vesicle Population to Synaptic Depression in the Tergotrochanteral and Dorsal Longitudinal Muscles of *Drosophila*

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Koenig, J. H. and Kazuo Ikeda. Relationship of the reserve vesicle population to synaptic depression in the tergotrochanteral and dorsal longitudinal muscles of *Drosophila*. *J Neurophysiol* 94: 2111–2119, 2005. First published June 15, 2005; 10.1152/jn.00323.2005. We have previously demonstrated that *Drosophila* synapses possess two vesicle populations—a small active zone population replenished by “fast” recycling and a much larger reserve population replenished by a slower recycling mechanism that includes endosomal intermediates. In this paper, we demonstrate that the synapses onto the tergotrochanteral muscle (TTM) are very unusual in that they possess only the active zone vesicle population but not the reserve population. The depression characteristics to repetitive stimulation of the TTM were compared with those of the dorsal longitudinal muscle (DLM), the synapses of which possess both an active zone and a reserve population. It was observed that the TTM response depressed more quickly than that of the DLM. To further explore the possible contribution of the reserve population to release, using the *shibire* mutant, DLM synapses were experimentally constructed that possess only the active zone population, and their depression characteristics were compared with those of the same synapses possessing both populations. It was observed that responses from DLM synapses possessing only the active zone population depressed more quickly than the same synapses possessing both populations. These experiments were conducted under conditions of blocked recycling so that the difference in stimulation tolerance represents the contribution of the reserve population to release. Furthermore, the depression curve of the DLM synapses lacking a reserve population now closely approximated that of the TTM synapses. These data suggest that the reserve vesicle population of DLM synapses may contribute to transmitter release during repetitive firing at physiological frequencies (5–10 Hz).

**INTRODUCTION**

In insects of higher order, two distinct types of skeletal muscle—fibrillar and tubular—have been distinguished based on morphological characteristics, such as differences in the arrangement of the actin/myosin complex, positioning of mitochondria and nuclei, the pattern of the sarcoplasmic reticulum, and the fibrillar sarcostyle. In *Diptera*, fibrillar muscle is confined to the dorsal longitudinal and dorsoventral flight muscles (DLMs and DVMs), whereas the other skeletal muscle in the fly is tubular. Tubular muscle has been termed “fast” muscle because of its relatively rapid rate of contraction, whereas fibrillar muscle has been termed “slow” because it is characterized by a slower rate of contraction (Tiegs 1955). These observations correlate well with the function of these muscles in the natural situation in that the fibrillar muscle tends to fire relatively slowly at a rate of ~5–10 Hz during flight, whereas tubular muscle fires rapidly and intermittently, providing movements such as walking or jumping.

Although the nature of the contractile response of these two types of muscle has been studied, the characteristics of synaptic transmission to these two different muscle types has not been well investigated. In this paper, we investigate, using intracellular recording, the electrophysiological response characteristics of the neuromuscular junctions (NMJs) of the tergotrochanteral muscle (TTM), a *Drosophila* tubular muscle responsible for the lift-off jump at flight initiation. The TTM response characteristics are compared with the response characteristics of the NMJs of the dorsal longitudinal flight muscle (DLM), a fibrillar muscle.

Based on the contractile properties of the two muscles, it has been assumed that the “fast” TTM should be able to tolerate high-frequency stimulation better than the slower DLM. It has been shown that with stimulation at the neck, the TTM pathway can follow high-frequency stimulation for several stimuli >200 Hz, whereas the DLM pathway fails at >100 Hz (Tanouye and Wyman 1980). It has also been shown that high-frequency stimulation of the TTM from the brain is less efficient than stimulating at the level of the thorax (Godenschwege et al. 2002). However, these studies do not address the capability of the TTM and DLM motor neurons themselves to release transmitter in response to high-frequency stimulation. In the present study, it was found that the DLM demonstrated less synaptic depression than did the TTM when stimulated for prolonged periods of time at all of the stimulation frequencies tested (from 10 to 100 Hz).

To find a possible explanation for the observed differences in synaptic depression between the TTM and DLM, the innervation and synapses of the TTM were observed using light and electron microscopy. It was discovered that the TTM synapses are very unusual in that they possess only a small “active zone” vesicle population made up of docked vesicles and vesicles closely tethered (within 150 nm) to the dense body at the active zone. This is very unusual because our previous work has shown that the DLM, as well as other skeletal tubular muscles such as the coxal or cervical muscles, all possess two vesicle populations—the small “active zone” population and a much larger reserve or nonactive zone population dispersed throughout the terminal cytoplasm away from the active zone. These two populations were distinguished by the fact that they are recycled by two completely different recycling pathways, emanating from different locations on the plasma membrane, with...
different time courses and sensitivities to Ca\(^{2+}\) (Koenig and Ikeda 1996).

The fact that the TTM synapses possess only the active zone population suggests that the reason they depress so quickly in response to repetitive stimulation might be related to the lack of a reserve population. To investigate this possibility, DLM synapses were experimentally constructed that possessed only an active zone population (similar to the TTM synapses), and the depression characteristics of these synapses were compared with those of the TTM. To construct these DLM synapses, the temperature sensitive endocytosis mutant, *shibire* (*shi*), was used. This mutant blocks recycling of synaptic vesicles at 29°C but exhibits normal recycling at 19°C (Koenig et al. 1983). The transmitter release mechanism is unaffected by the mutation (Ikeda et al. 1976), and the muscle response is also unaffected (Koenig and Ikeda 1996). We have previously shown that if *shi* DLM synapses are exposed to 29°C while stimulating, complete vesicle depletion occurs as exocytosis proceeds normally while endocytosis is blocked (Koenig et al. 1989). We have further shown that on returning the depleted synapses to 19°C, the small, active zone vesicle population completely reforms before the reserve population begins to reform (Koenig and Ikeda 1996). In this way, a fly can be experimentally constructed that possesses only the active zone population at its DLM synapses (Koenig and Ikeda 1999). Such a fly was made, and the response characteristics of the DLM synapses possessing only the active zone population were compared with the response characteristics of the same DLM synapses possessing both populations as well as with those of the TTM that possess only the active zone population. It was observed that the DLM depressed more quickly when the DLM synapses possessed only the active zone population and approximated the TTM depression response. Thus our results suggest that in the DLM, the reserve population may contribute to release, even at relatively low firing frequencies such as those observed under normal flight conditions.

**Methods**

Four-day-old female *Drosophila melanogaster* of the wild-type strain, Oregon-R, and the single gene recessive, temperature-sensitive mutant, *shibire* ts1 (*shi*), were used for these experiments. The *shi* gene encodes the protein, dynamin (Chen et al. 1991; van der Bliek and Meyerowitz 1991), which is involved in the process whereby invaginations of the plasma membrane pinch off to form vesicles or cisternae (Baba et al. 1995; Danke et al. 1994; Hinshaw and Schmid 1995; Kosaka and Ikeda 1983, a; Takei et al. 1995). The mutant dynamin functions normally in *shi* synapses at 19°C but becomes dysfunctional at 27°C possibly due to a configurational change in the molecule. Thus with stimulation at 29°C, vesicle depletion occurs because vesicle recycling is blocked. When the temperature is returned to 19°C, vesicle recycling is restored and the vesicle population is reformed by two distinct recycling pathways.

**Light microscopy of the TTM innervation**

For details of the light microscopic dissection, see Ikeda and Kaplan (1970). Briefly, the fly was immobilized on its side in Tackiwax over a plastic tube, allowing the underside to be exposed to air in the tube for respiration, then immersed in saline containing (in mM) 128 NaCl, 4.7 KCl, 1.8 CaCl\(_2\), and 5 Tris aminomethane HCl (pH 7.4). The right wing and lateral cuticle overlying the TTM were removed. The fly was then fixed by replacing the saline with aequous Bouin’s solution, and the preparation was processed for silver impregnation as described in Ikeda et al. (1980).

**Electron microscopy of the TTM synapses**

For details of the electron microscopic techniques, see Koenig et al. (1983). Briefly, the fly was dissected in saline to expose the lateral surface of the TTM fibers. The saline was instantly replaced by fixative (2% paraformaldehyde, 2% gluteraldehyde in 0.1 M phosphate buffer, pH 7.4 for 30 min, followed by 4% gluteraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h), after which the fly was 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 Spurr’s medium. Thin sections of white interference color were poststained with 2% uranyl acetate in 70% ethanol and Millonig’s lead hydroxide solution, and were observed with a Philips CM-10 electron microscope and photographed.

**Intracellular recording from TTM and DLM**

The fly was immobilized on its left side in Tackiwax, securing the thorax but allowing the abdomen to move freely so as to allow natural breathing through the spiracles. After cutting off the right wing, a droplet of saline was continuously applied to the lateral surface of the cuticle via a small plastic tube filled with saline, and the lateral cuticle was removed to expose the TTM. Care was taken not to disturb the dorsal cuticle where the TTM attaches. For intracellular recording, a glass micropipette filled with 0.5% Niagara Sky Blue dye in distilled water (for visibility) was inserted through the dorsal cuticle into a TTM fiber at its attachment site to the cuticle. A second micropipette filled with dye was then inserted through the dorsal cuticle into DLM fiber 5 or 6 at its attachment site. With proper lighting, the attachment sites of the fibers were visible through the cuticle. The ground electrode, a fine silver wire, was inserted into the droplet of saline, and the stimulating electrode was inserted into the giant interneuron at the neck. Both the TTM and DLM receive input from this interneuron (Bacon and Strausfeld 1986; Tanouye and Wyman 1980). The interneuron was stimulated with a 0.1-ms square pulse at various frequencies. With this preparation, it was possible to stimulate and record simultaneously from the TTM and DLM in the same fly. In those experiments where the electrogenic (voltage-dependent) component of the response was suppressed, application of the 4 mM Na\(^+\)-glutamate in saline was accomplished through the plastic tube.

For details of the recording technique for the DLM of *shi* flies, see Koenig et al. (1983) and Ikeda and Koenig (1988). Briefly, while the temperature was being maintained at 19°C, the fly was mounted in Tackiwax over an opening in a plastic tube so that its underside could remain exposed to the air in the tube while the fly was submerged in saline. The DLM were exposed by dissecting away the mesothoracic preepisternum, pleura, dorsoventral muscles, and the tergrotrochanteral muscle. The recording electrode was inserted through the dorsal cuticle into the DLM fiber, and the ground electrode was in the saline. The posterior dorsal mesothoracic nerve (PDMN) that contains the five axons innervating the DLM was cut, and the distal end of the nerve was sucked into a suction electrode for stimulation. Cutting the PDMN was necessary to eliminate the spontaneous ganglionic activity that is triggered in *shi* flies as the temperature is raised to >26°C. The experiments were performed in glutamate saline to suppress the electrogenic (voltage-dependent) component of the response. The temperature was raised by instantly replacing the 19°C saline with 29°C saline. The temperature was monitored by a thermometer placed in the bath as near to the fly as possible. Only those flies that maintained a DLM resting potential of ~90 mV or more were used for these results.

For the *shi* TTM recordings, the fly was mounted in Tackiwax as described in the preceding text, submerged in saline, and dissected to expose the TTM. The recording electrode was inserted through the
dorsal cuticle, and the TTM motor neuron was stimulated as described in the preceding text by an electrode inserted into the neck. No spontaneous ganglionic activity was observed in the TTM when the temperature was raised. The experiments were performed in glutamate saline.

RESULTS

Innervation of the TTM

The tergotrochanteral muscle spans dorsoventrally in the lateral part of the mesothorax, inserting dorsally at the lateral tergum and ventrally at the trochanter of the mesothoracic leg. It is composed of 22–29 tubular muscle fibers that are arranged circularly, forming a monolayer cylinder. This arrangement is quite unusual among tubular muscles. Because its dorsal insertion creates an antero-posteriorly oriented oval approximately 200 μm long, whereas the ventral insertion is a smaller compact circular shape, the lateral view of the muscle is fan-shaped as is shown diagrammatically in the longitudinal section of the thorax shown in Fig. 1A. When this muscle contracts, it causes the second leg to extend rapidly, thereby providing the jumping response used to launch the fly at flight initiation (Mulloney 1969; Trimarchi and Schneiderman 1995a).

Three axons—one giant axon ~5 μm in diameter and two fine axons ~1 μm in diameter—were observed to innervate the TTM. In this paper, the larger axon will be referred to as the giant motor axon and the other two as the fine axons. One large and two fine axons have also been reported as innervating the TTM in another species of diptera, Musca (Bacon and Strausfeld 1986). The nerve bundle composed exclusively of these three axons branches off from the posterior dorsal mesothoracic nerve (PDMN) ~30 μm from where the PDMN exits the thoracic ganglion. This bundle will be referred to as the tergotrochanter nerve (TTN) in this paper. The TTN runs toward the anterior-median corner of the TTM at a level of about one-fourth of the muscle length from the trochanter insertion, and after entering the muscle, the giant axon separates from the two fine axons, which run anteriorly and each innervate two particular muscle fibers located at the anterior median corner of the muscle (Fig. 1B). In Fig. 1, C and D, two cross-sections of the TTN, one as it approaches the TTN (C) and the other as it enters the TTM (D), demonstrate the giant motor and the two fine axons. In this paper, we focus on the innervation of the TTM by the giant motor axon, which provides the input for the jump response. The innervation of the fine axons will appear in a separate publication.

After separation from the fine axons, the giant motor axon travels further inside of the muscle bundle until it reaches the center of the monolayer cylinder created by the muscle fibers, where it bifurcates into dorsal and ventral branches (Fig. 2 A–D). These dorsal and ventral branches maintain their large diameter and serve as the main trunk off of which come many fine branches at various levels. The pathway of the main trunk of the giant motor axon running parallel to the muscle fibers in the central core of the muscle is unique to the TTM. Other tubular muscles of Drosophila so far studied in our laboratory are innervated by axon(s) running on the outer surface of the muscle fibers rather than inside the muscle.

The main trunk runs parallel to the muscle fibers and sends out fine branches perpendicular to itself at intervals of ~50 μm (Fig. 2 E and F). These fine branches come out of the main trunk at different levels and also at different angles covering 360°, creating a spiral staircase effect, as the branches innervate the various different fibers making up the cylinder (with the exception of the four fibers innervated by the 2 fine axons). Each fine branch travels in between two of the muscle fibers and innervates those fibers by making en passant synapses on both sides.

Electrophysiological response of TTM

The electrical responses of the TTM fibers were recorded intracellularly by inserting a fine glass micropipette through the dorsal cuticle and directly into the fiber where it attaches to the tergum. The TTM motor neuron was stimulated by passing current through a microelectrode inserted into the giant interneuron at the neck. (See METHODS for full description of recording conditions.) A typical response obtained by intracellular recording from a TTM fiber is seen in Fig. 3A. The response amplitude was about ~85 mV and appeared to possess an excitatory junction potential (EJP) and a voltage-
muscle fibers. Silver impregnated specimen at 2 different planes of sectioning, parallel to the arrowhead, branch of main trunk of giant axon. Scale bar for of muscle fibers. Large arrow, giant axon trunk; small arrow, fine axon; can be seen.

The main dorsal trunk (large arrow) moves more toward the center of the TTM while extending a fine branch (arrowhead). In this section, the 2 fine axons (small arrows) can be seen.

Electron microscopy of TTM synapses

To observe the synaptic depression characteristics of the TTM response and compare them with those of the DLM, the TTM and DLM motor neurons were stimulated ≥250 times at various frequencies. Simultaneous recordings from the TTM and DLM fibers were made in the same fly. In Fig. 4, the depression curves of the TTM and DLM at 10, 20, 50, and 100 Hz are shown. Each of the curves represents an average of depression curves from five different flies. The initial spike amplitudes for these recordings were very similar as were the depression characteristics, so that the maximum SD for each of these averaged curves was never above ±0.1 (See figure legend for specific SDs). From this figure, it becomes apparent that at any stimulation frequency, the TTM does not tolerate repetitive stimulation as well as the DLM. Also it can be observed that as the frequency of stimulation is increased, the depression curve appears to display two phases, an initial phase in which the amplitude reduces quickly (large dv/dt) followed by a phase in which the amplitude reduces much more slowly (small dv/dt) and levels off. At the higher stimulation frequencies (Fig. 4D), the depression curve of the DLM more closely approximates that of the TTM in its initial steep phase, but the amplitude at which the response levels off differs.

The primary cause of short-term synaptic depression is thought to be a limitation in the availability of readily releasable vesicles. To find a possible explanation for the unexpected poorer tolerance to repetitive stimulation of the TTM relative to the DLM, the synapses of the giant motor axon onto the TTM were observed using electron microscopy, paying particular attention to the morphology of the active zone (readily releasable) vesicle population.
The synapses were located on the fine side branches made by the giant motor axon as described above. These side branches were found to be amazingly perpendicular to the main large trunk of the giant axon, which allowed visualization of a long section of the branch in one EM section (Fig. 5). The initial few micrometers of the fine branch are covered by a glial sheath (arrow in Fig. 5A), but the rest of the branch is free of the glial sheath. At the point where the branch becomes naked, en passant type synapses can be seen almost every 1 μm on the membranes of the muscle fibers on either side of the branch (arrowheads in Fig. 5A). A single muscle fiber is composed of ~160 sarcostyles (4 layers of ~40 sarcostyles along the innervating axon branch), and these are separated from each other by the transverse tubular system (sarcoplasmic reticulum). Every sarcostyle appears to receive one synaptic contact.

The synapses possessed the typical dense body for *Drosophila*, and the typical small active zone population of synaptic vesicles, which are either docked or closely tethered to the dense body (Fig. 5B). The average number of vesicles/active zone/plane of sectioning for 50 active zones/fly was calculated for three flies. The averages (with SD) were: 10.6 ± 8.2, 9.9 ± 8.0, and 10.3 ± 8.6. These averages are equivalent to those previously obtained for the DLM (Koenig and Ikeda 1999; Koenig et al. 1989). Thus morphologically, the active zones of the DLM and TTM were indistinguishable.

We have shown previously that the active zone vesicle population mentioned in the preceding text is recycled by direct pinch-off from the presynaptic membrane at the active zone and includes both docked vesicles and a small cluster of tethered vesicles above the dense body plate (Koenig and Ikeda 1996). With fortuitous planes of sectioning through an active zone, the fine fibrils to which the vesicles above the plate are attached can be imaged (see Fig. 1D, Koenig et al. 1998), but in many cases, these fibrils appear in cross-section and cannot be seen. Therefore the active zone vesicles are identified by
two criteria: 1) the location of the vesicles (within 150 nm of the dense body); and 2) the recycling pathway from which they were formed. Because the reserve vesicle population is formed by a completely different recycling pathway with a different time course and emanates from presynaptic membrane away from the active zone, it is possible to distinguish the two populations in this way. One striking difference between the TTM and DLM synapses was immediately apparent. Unlike other NMJs so far observed in Drosophila, the TTM synapses did not possess the larger reserve population of vesicles that is normally present in the cytoplasm away from the active zone. An example of the difference between the TTM NMJs and those of another tubular muscle, the pleurosternal muscle, is shown in Fig. 6. As can be seen in Fig. 6A, the pleurosternal synapses possess a very large reserve population dispersed throughout the cytoplasm, in addition to the smaller population at the active zones (arrows). By contrast, the cytoplasm away from the active zone of the TTM synapses is devoid of vesicles (Fig. 6B). Electron microscopic images of other NMJs demonstrating the reserve population can be seen in Koenig and Ikeda (1999) (DLM) and Koenig and Ikeda (1989) (coxal, cervical muscles).

Contribution of reserve population to transmitter release

The fact that the TTM NMJs do not possess a reserve vesicle population suggests a possible explanation for why the TTM response depresses more quickly than the response of the DLM, which does possess a reserve population (for DLM reserve population, see Koenig and Ikeda 1999). For example, if in addition to “fast” recycling, the reserve population of the DLM was contributing to the replenishment of the readily releasable population in a significant way during stimulation, this might reduce the rate of synaptic depression in the DLM (relative to the TTM).

To explore this possibility, the depression characteristics of shi TTM synapses were compared with those of shi DLM synapses possessing only an active zone vesicle population. The DLM synapses possessing only the active zone vesicle population were constructed as follows: the temperature was raised to 29°C to block recycling, and the DLM response was recorded while stimulating at 10 Hz until the EJP amplitude reached the failure level, i.e., until the synapses were completely depleted of vesicles. Then, the temperature was lowered to 19°C, allowing vesicle recycling to begin. We have shown previously by electron microscopy and electrophysiology that in the DLM NMJs, the active zone population is fully reformed within ~1 min, whereas the reserve population does not even begin to form recycling cisternae until ~2 min (Koenig and Ikeda 1999). Therefore after 1.5 min at 19°C, the temperature was again raised to 29°C to block any further recycling, resulting in a fly with DLM synapses possessing only the active zone vesicle population.

To observe the initial depression of the EJP without the voltage-dependent component, in these experiments the synapses were exposed to saline containing 4 mM Na₃-glutamate to bring the amplitude of the EJP to below firing level. DLM fibers 5 and 6 were used for these experiments. To block the spontaneous ganglionic activity that occurs in the DLM motor pathway in shi at 26–29°C, the PDMN was cut and sucked into a suction electrode and the DLM motor axons were stimulated directly.

The DLM motor neuron was stimulated at 10 Hz and recorded a second time at 29°C. Two depression curves from the same synapses to the same muscle were obtained from this experiment—one during the first stimulation at high temperature (with both active zone and reserve populations present) and a second during the second stimulation at high temperature (with only the active zone population present in the terminals).

After stimulating for a second time, the flies were returned to 19°C, and recycling was allowed to proceed. We have shown previously that both vesicle populations become completely replenished after ~30 min at 19°C (Koenig and Ikeda 1999). After 40 min exposure to 19°C, a third depression curve at 29°C was taken to assure that the condition of the experimental animal had not contributed to a reduction in stimulation tolerance. Only those flies that demonstrated a full recovery, with a third depression curve equivalent to the first depression curve taken when both vesicle populations were present in the terminals, were used in this study.

As a second control, some shi flies were fixed for EM after the DLM synapses had been depleted at 29°C and returned to 19°C to allow the active zone population to recycle for 1.5 min. This was to verify that the synapses indeed possessed normal active zone populations of vesicles but lacked the reserve population. Forty randomly selected synapses were observed in each of seven flies and the number of active zone vesicles/active zone/plane of sectioning was noted. The average number of vesicles was 10.8, which, as mentioned in the preceding text, has previously been shown to be the average number of vesicles in a wild-type active zone. No reserve vesicles were observed.

For the TTM depression curves under conditions of blocked recycling, shi flies were exposed to 29°C, and the TTM was recorded intracellularly while stimulating at 10 Hz. These preparations were also exposed to glutamate saline to eliminate the electrogenic component.

The data from the DLM and TTM recordings are presented in Fig. 7. As can be seen, the DLM synapses possessing both
populations exhibited a higher tolerance to repetitive stimulation than the same synapses possessing only the active zone population. The DLM depression curves in Fig. 7 represent an average from six different flies. Again, the results from different flies were amazingly consistent, so that the SD never exceeded 0.12. The two DLM depression curves in Fig. 7 demonstrate that there is a difference in the rate of short-term synaptic depression between DLM synapses with and without a reserve vesicle population. Because the data were taken under conditions of blocked recycling, this difference must reflect the contribution of the reserve population to the readily releasable pool of vesicles.

The third depression curve in Fig. 7 represents the averaged results from TTM synapses in seven different shi flies at 29°C, i.e., under conditions of blocked recycling. If this third curve is compared with the second shi DLM curve (from synapses in which no reserve population is present under conditions of blocked recycling), it can be seen that the DLM depression characteristics now more closely approximate those of the TTM. However, the shi TTM synapses still consistently depressed at a slightly faster rate than the DLM, even when both DLM and TTM synapses possessed only the active zone vesicle population. Because no recovery of the reserve population has ever been observed to occur at 29°C, recycling being completely blocked, this suggests that another factor in addition to the lack of a reserve population must be affecting the difference in depression curve rates between the TTM and DLM.

As can be seen in Fig. 7, the “fast synaptic fatigue” previously reported for shi DLM (Kawasaki et al. 2000) was not observed under our recording conditions. Even at 100 Hz stimulation, we have never observed a significant drop in EJP amplitude within 20 ms of the first response as reported by Kawasaki et al. (2000) in either wild-type (Fig. 4C) (also Tanouye and Wyman 1980) or shi DLM synapses.

**Discussion**

We have previously shown using electron microscopy of the shi mutant that Drosophila synapses possess two different recycling pathways that form two different synaptic vesicle populations. The small active zone population is composed of vesicles docked under the dense body at the active zone and vesicles closely tethered to the dense body (within 150 nm) and is replenished by a “fast” recycling pathway mediated by direct pinch-off of vesicles from the presynaptic membrane at the active zone. The much larger non-active zone or reserve population is composed of vesicles dispersed throughout the terminal cytoplasm away from the active zone and is replenished by a slower recycling pathway mediated by endosomal intermediates (Koenig and Ikeda 1996). Subsequently, the existence of two vesicle populations with two different recycling pathways in a synapse has been reported in various other organisms as well (e.g., Richards et al. 2000, frog; Teng and Wilkinson 2000, snake; de Lange et al. 2003; Kumashiro et al. 2005, rat; Van der Kloot 2003, Torpedo).

In this paper, the synapses of the TTM are described and are shown to be very unusual in that they possess only the small active zone population but not the larger reserve population typically observed in other NMJs and neuro-neuronal synapses so far studied in Drosophila. Prolonged repetitive stimulation demonstrated that the TTM exhibited greater synaptic depression than did the DLM, a muscle with synapses that possesses a reserve vesicle population, which led us to investigate the possibility that these differences in the degree of synaptic depression might be the result of the contribution of the reserve population to release during repetitive stimulation. To explore this possibility, DLM synapses were created using the shi mutant that lack a reserve population to see if the depression characteristics would become similar to those of TTM synapses, which naturally lack a reserve population. This was found to be the case. The DLM depression curve much more closely approximated the TTM depression curve when the DLM synapses did not possess a reserve population. In addition, the same DLM synapses in the same fly with or without a reserve population were compared. These experiments were performed in a condition of blocked recycling (29°C), so that any difference in the degree of depression would have to be the result of mobilization of the reserve population. It was observed that the DLM synapses lacking the reserve population exhibited greater depression than did the same synapses possessing both populations.

In a previous paper using the shi mutant, we created a DLM innervated by synapses possessing only an active zone population and reported that the response to repetitive stimulation at physiological frequencies (5–10 Hz) was equivalent to that of a DLM possessing both populations (Koenig and Ikeda 1999). This observation was made at 19°C, so that active zone recycling (“fast” recycling) was apparently able to provide the synapses with enough transmitter to bring the responses above threshold for firing, thus sustaining normal activity. This result immediately brought into question the contribution to synaptic transmission of the reserve population. Certainly it demonstrated that the reserve population was not necessary for prolonged firing at physiological frequencies. Other studies have also demonstrated a minor role in transmission for the reserve population. For example, in frog NMJs, it has been reported that transmitter release during low-frequency stimulation (2–5 Hz) is contributed almost exclusively if not entirely by the readily releasable population (Richards et al. 2003). In the rat calyx of Held, at physiological frequencies (5 Hz), a
small fast recycling vesicle pool provides enough vesicles for normal release (de Lange et al. 2003). In hippocampal synapses, it has been shown that fast vesicle recycling and vesicle reuse, rather than the reserve vesicle population, are primarily responsible for maintaining the population of releasable vesicles even with intense stimulation (30 Hz) (Sara et al. 2002). In Drosophila larval NMJs, it has been reported that a smaller, more releasable population ("exo/endo cycling") provides transmitter at lower frequencies and that the reserve population only comes into play during tetanic stimulation (Kuromi and Kidokoro 2000). It has been further reported that this exo/endo cycling population may include two pools, an "immediately releasable pool (IRP)" and a "readily releasable pool (RRP)" (Delgado et al. 2000). These two pools may coincide with the docked vesicles (IRP) and the tethered vesicles (RRP) observed in the active zone population of the TTM synapses.

The data presented here seem to indicate that in the DLM, the reserve population may be mobilizing and contributing in a significant way to the readily releasable pool of vesicles during repetitive stimulation at physiological frequencies even though it has been shown previously that with active zone (fast) recycling, the reserve population is not necessary to maintain a normal response with repetitive firing, at least for a limited period of time (Koenig and Ikeda 1999). A contribution by the reserve population under normal firing conditions is an unexpected finding, but if one considers the physiological requirements of the DLM (steady, prolonged firing during flight), it seems reasonable that a mechanism might be in place that allows the reserve population to play a more prominent role during sustained activity than in other muscles that do not engage in prolonged firing.

Under conditions of blocked recycling, it was observed that the TTM synapses depressed much more quickly than did those of the DLM possessing both active zone and reserve vesicle populations. However, when the depression curve of DLM synapses lacking a reserve population is compared with that of TTM synapses (naturally lacking a reserve population), the depression characteristics of the two different muscles become quite similar. Thus the presence of a reserve population might explain why the DLM can tolerate stimulation significantly better than the TTM. It was observed, however, that the TTM consistently depressed slightly more rapidly than the DLM even though the DLM had no reserve population. One possible factor that might cause this difference is that the DLM possesses many more active sites than the TTM.

The morphological and physiological differences between these two types of synapses are consistent with the different firing requirements of these muscles in the natural situation. The jumping movement at flight initiation provided by the TTM is a single event and is followed by inactivity as the flight proceeds. In fact, it was shown that the TTM fires only once at the start of flight, regardless of triggering mode (visual, olfactory stimulation, or voluntary) (Trimarchi and Schneiderman 1995b). Furthermore, it has been shown in Musca that the TTM fatigues rapidly from repetitive stimulation (Schouest et al. 1986). Thus it seems reasonable to assume that a readily releasable (active zone) vesicle population would be the only population needed for this single event. This is an unusual situation for most tubular muscles, that are required to fire for longer periods of time in activities such as walking, eating, or preening. The firing requirement of the DLM, on the other hand, is also unusual in that it requires steady, prolonged firing. Such long-term sustained activity might put a strain on the fast recycling pathway to replenish the readily releasable pool. A reserve population that contributed during long-term firing would help relieve this strain.

The contrasting characteristics of NMJs involved in phasic and tonic activity have been well studied in crustacea (see review by Atwood and Karumanithi 2002). Phasic muscle, involved in short-term intermittent firing similar to the TTM, tends to demonstrate depression, whereas tonic muscle, involved in more repetitive firing similar to the DLM, demonstrates facilitation. However, unlike the DLM and TTM synapses described here, the crustacean synapses to both phasic and tonic muscle possess both readily releasable and reserve vesicle populations, and the differences in response are attributed to differences in release probability (phasic, high probability; tonic, low probability).

In conclusion, these data show that the TTM NMJs are unusual in not possessing a reserve vesicle population and also demonstrate greater degree of synaptic depression in response to repetitive stimulation relative to the DLM, which does possess a reserve population. The data are consistent with the possibility that the presence of a reserve population in DLM NMJs may explain, at least in part, why this muscle shows a lesser degree of depression than that of the TTM, which lacks a reserve population.

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