Properties of Short-Term Synaptic Depression at Larval Neuromuscular Synapses in Wild-Type and Temperature-Sensitive Paralytic Mutants of *Drosophila*

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Wu, Ying, Fumiko Kawasaki, and Richard W. Ordway. Properties of short-term synaptic depression at larval neuromuscular synapses in wild-type and temperature-sensitive paralytic mutants of *Drosophila*. *J Neurophysiol* 93: 2396–2405, 2005; doi:10.1152/jn.01108.2004. The larval neuromuscular synapse of *Drosophila* serves as an important model for genetic and molecular analysis of synaptic development and function. Further functional characterization of this synapse, as well as adult neuromuscular synapses, will greatly enhance the impact of this model system on our understanding of synaptic transmission. Here we describe a form of short-term synaptic depression observed at larval, but not adult, neuromuscular synapses and explore the underlying mechanisms. Larval neuromuscular synapses exhibited a form of short-term depression that was strongly dependent on stimulation frequency over a narrow range of low frequencies (0.1–1 Hz). This form of synaptic depression, referred to here as low-frequency short-term depression (LF-STD), results from an activity-dependent reduction in neurotransmitter release. However, in contrast to the predictions of depletion models, the degree of depression was independent of the initial level of neurotransmitter release over a range of extracellular calcium concentrations. This conclusion was confirmed in two temperature-sensitive (TS) paralytic mutants, *caкопhony* and *shibire*, which exhibited reduced neurotransmitter release resulting from conditional disruption of presynaptic calcium channels and dynamin, respectively. Higher stimulation frequencies (40 or 60 Hz) produced two components of depression that appeared to include LF-STD as well as a more conventional component of short-term depression. These findings reveal novel properties of short-term synaptic depression and suggest that complementary genetic analysis of larval and adult neuromuscular synapses will further define the in vivo mechanisms of neurotransmitter release and short-term synaptic plasticity.

**INTRODUCTION**

A key feature of chemical synaptic transmission is its plasticity, which allows the functional strength of a synaptic connection to be modified by neuronal activity (Bear 2003; Ito 2002; Lynch 2004; Zucker and Regehr 2002). Synaptic plasticity is a primary mechanism underlying experience- or activity-dependent changes in the nervous system and thus contributes to virtually all aspects of neural function. Synaptic strength may be increased (facilitation, augmentation or potentiation) or decreased (depression), and these changes may be either transient (short term) or persistent (long term).

The present study is focused on short-term depression. When stimulated repetitively under physiological conditions, many synapses exhibit rapid depression that progresses to a steady-state level and recovers in seconds after stimulation. Although the underlying mechanisms remain a matter of intensive study and debate (Zucker and Regehr 2002), it is generally agreed that many forms of short-term depression reflect activity-dependent changes in neurotransmitter release. Because this activity dependence lies at the heart of models defining stages of the synaptic vesicle trafficking cycle, including vesicle docking, priming, fusion and recycling, further genetic and molecular analysis of short-term depression will make important contributions to our understanding of neurotransmitter release mechanisms.

One powerful approach has involved genetic analysis in *Drosophila* to investigate the in vivo molecular mechanisms of synaptic function. Much of this work has been carried out at neuromuscular synapses of identified body wall muscles in the embryo and third instar larva as well as neuromuscular synapses of the adult. The embryonic and larval preparations have been characterized extensively and serve as important models for analysis of synaptic development, plasticity, and function (Keshishian et al. 1996; Koh et al. 2000; Matthies and Broadie 2003; Richmond and Brodie 2002; Rose and Chiba 2000; Stimson and Ramaswami 1999; Wu and Bellen 1997). Adult neuromuscular preparations have been utilized for investigating mechanisms of synaptic function, for example in defining the roles of dynamin (Ikeda et al. 1976; Kawasaki and Ordway 2000; Koenig and Ikeda 1996; Kosaka and Ikeda 1983; Fodory and Edgar 1979; Salkoff and Kelly 1978), NSF (Kawasaki et al. 1998), and presynaptic calcium channels (Brooks et al. 2003; Kawasaki et al. 2000, 2002, 2004), and important developmental studies have been carried out as well (Fernandes and Keshishian 1999, 1998; Ikeda and Koenig 1988; Rivlin et al. 2004; Sun and Wyman 1997; Trimarchi et al. 1999).

Functional analysis at larval neuromuscular synapses has extended to investigation of short-term synaptic depression (Delgado et al. 2000; Renger et al. 2000; Zhong and Wu 1991), and recent work has begun to define this process at adult neuromuscular synapses (Kawasaki and Ordway 2000). Despite this progress, further characterization of short-term depression is needed to facilitate direct comparison with other systems and realize the full potential of *Drosophila* as a general model for analysis of synaptic development, plasticity, and function.

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model for analysis of neurotransmitter release. Moreover, the properties of short-term depression at larval and adult neuromuscular synapses may reveal instructive differences. Here we report a distinct form of short-term synaptic depression at larval neuromuscular synapses, low-frequency short-term depression (LF-STD), defined by its sensitivity to low-frequency stimulation, lack of dependence on the initial level of neurotransmitter release, and slow recovery. Furthermore, the process responsible for LF-STD also appears to participate in short-term depression at high stimulation frequencies along with a second, more conventional, component of depression. The properties of LF-STD are compared with those observed in other preparations as well as previous work at larval neuromuscular synapses. Finally, potential mechanisms of LF-STD are discussed.

**METHODS**

**Fly stocks**

*cac*^T32^ (Dellinger et al. 2000), *shi*^T51^ and *shi*^T32^ (Grigliatti et al. 1973) stocks were from our laboratory stock collection. Wild-type flies were Canton S.

**SYNAPTIC ELECTROPHYSIOLOGY.** Third-instar larvae were maintained at 20°C and analyzed at 20°C or the indicated temperature. Larvae were dissected and the nerves projecting from the ventral ganglion cut as described previously (Jan and Jan 1976). Two-electrode voltage-clamp recordings of synaptic currents were carried out using a TEV-200 amplifier (Dagan, Minneapolis, MN) as described previously (Kawasaki et al. 1998). Synaptic currents were recorded from neuromuscular synapses of ventral longitudinal muscle 6 in abdominal segment A2 or A3 at a holding potential of −60 mV. Electrode voltage-clamp recordings of synaptic currents were carried out using a TEV-200 amplifier (Dagan, Minneapolis, MN) as described previously (Kawasaki et al. 1998). Two-electrode voltage-clamp recordings of synaptic currents were carried out using a TEV-200 amplifier (Dagan, Minneapolis, MN) as described previously (Kawasaki et al. 1998). Synaptic currents were recorded from neuromuscular synapses of ventral longitudinal muscle 6 in abdominal segment A2 or A3 at a holding potential of −60 mV. Deviations from the command potential typically did not exceed 2 mV. Unless indicated otherwise, all experiments were conducted in a standard recording solution containing (in mM): 128 NaCl, 2 KCl, 4.0 MgCl$_2$, 1.8 CaCl$_2$, 36 sucrose, and 5 HEPES (pH 7.0). Solutions containing different calcium concentrations were generated by adjusting the molarity of CaCl$_2$. Hemolymph-like (HL-3) solution was prepared as described (Stewart et al. 1994). Temperature control and motor axon stimulation were achieved as described previously (Kawasaki et al. 1998). Glass microelectrodes were filled with 3 M KCl. Recordings from one or two muscle fibers were obtained from each preparation. Unless stated otherwise, n refers to the number of muscle fibers analyzed.

**DATA ACQUISITION AND ANALYSIS.** Data were acquired on-line using Pulse software (Heka Electronic, Lambrecht, Germany) and an ITC-16 laboratory interface (Instrutech, Great Neck, NY). Data were typically low-pass filtered at 5 kHz and acquired at 25 kHz. Cursor measurements of synaptic currents and curve-fitting were carried out using the data analysis software package, IGOR (Wavemetrics, Lake Oswego, OR). Steady-state EPSC amplitudes were determined by averaging three EPSCs at the end of the stimulus train. Microsoft (Seattle, WA) Excel was used for analysis of numerical data, statistics and graphing. Data are reported as the means ± SE. Statistical significance was evaluated using the unpaired Student’s t-test and significant differences assigned to comparisons for which P < 0.05.

**RESULTS**

**Short-term depression at larval neuromuscular synapses**

To examine the short-term activity dependence of larval neuromuscular synaptic transmission, two-electrode voltage-clamp was employed to record EPSCs over a range of stimulation frequencies. Axonal stimulation at a low frequency of 0.05 Hz produced no clear depression or facilitation, rather the EPSC amplitude after 20 pulses was 97.0 ± 4.4% of the initial amplitude (n = 8). However, stimulation frequencies as low as 0.1 Hz produced substantial short-term depression. For example, 1-Hz stimulation decreased the EPSC amplitude to a steady-state level corresponding to 65.8 ± 3.4% (n = 16) of the initial amplitude (Fig. 1A). This sensitivity to low-frequency stimulation distinguishes larval neuromuscular synapses from those of the *Drosophila* adult, at which little or no synaptic depression was observed at 1 Hz (Kawasaki and Ordway 2000; Kawasaki et al. 1998).

At larval neuromuscular synapses, the degree of depression was strongly dependent on stimulation frequency over a narrow range of low frequencies (0.1–0.5 Hz; Fig. 1, B and C). At 0.1, 0.2, and 0.5 Hz, the respective steady-state EPSC amplitudes were 85.6 ± 1.4% (n = 10), 74.9 ± 1.8% (n = 9), and 68.7 ± 1.8% (n = 7) of the initial amplitude. In contrast, over a wide range of higher frequencies (1–20 Hz), the degree of depression was insensitive to frequency (Fig. 1C). At 1, 5, 10, and 20 Hz, the steady-state EPSC amplitudes were 65.8 ±

![Image](http://jn.physiology.org/10.1152/jn.00520.2005)  
**FIG. 1.** Short-term synaptic depression at larval neuromuscular synapses. A: representative excitatory postsynaptic current (EPSC) traces from the first 20 responses of a 1-Hz stimulation train are superimposed to illustrate progressive activity-dependent reduction in EPSC amplitude. Stimulation artifacts were removed for clarity. B: peak EPSC amplitudes as a percentage of the initial amplitude are plotted as a function of stimulus number at low stimulation frequencies (0.05, 0.1, and 0.5 Hz). C: the steady-state level of depression, expressed as the ratio of the steady-state and initial EPSC amplitudes (EPSC$_{ss}$/EPSC$_{o}$), is plotted vs. stimulation frequency. Hail-maximal depression was observed at 0.1–0.2 Hz. The degree of depression at 1 Hz was not significantly different from that at 5, 10, or 20 Hz. D: recovery of depression was examined by recording EPSCs evoked by a test pulse at different intervals after 1- or 20-Hz stimulation (100 pulses). For both frequencies, recovery was nearly complete at 20 s, consistent with the lack of significant depression at 0.05 Hz.
0.9% (n = 16), 65.6 ± 1.3% (n = 6), 63.1 ± 2.2% (n = 8), and 62.0 ± 1.8% (n = 8) of the initial value, respectively. To address whether the same mechanism produces depression over this range of frequencies, recovery from depression was examined and found to be similar after 1- and 20-Hz stimulation (Fig. 1D). These results indicate that larval neuromuscular synapses exhibit a form of short-term synaptic depression that is elicited by low-frequency stimulation, maximal at 1 Hz, and predominant over a wide range of frequencies (0.1–20 Hz). This process is referred to here as LF-STD. The present study defines the properties of LF-STD and examines its relationship to a second component of short-term depression elicited by high-frequency stimulation. Although distinctive, the characteristics of LF-STD appear to overlap with those of short-term depression observed in other systems.

LF-STD at larval neuromuscular synapses is a robust phenomenon occurring in different fly strains and recording conditions. Similar results were obtained in Canton S, shibire\textsuperscript{TS1} (shi\textsuperscript{TS1}) and cacophony\textsuperscript{TS2} (cac\textsuperscript{TS2}) strains over a wide range of temperatures and in another recording solution used for this preparation, HL-3 (Stewart et al. 1994) (data not shown). Furthermore, both identified neurons innervating muscle 6, the RP3 and 6/7b motor neurons (Jan and Jan 1976; Keshishian et al. 1996), exhibited clear LF-STD. This was assessed by selectively stimulating the lower threshold motor neuron to elicit steady-state LF-STD and then increasing the stimulus intensity to also recruit and depress the higher threshold unit (data not shown). The properties of LF-STD suggest that it may be expressed routinely under native conditions, in which contractile wave frequencies exceed 0.5 Hz during normal locomotion (Ainsley et al. 2003; Wang et al. 2002).

**LF-STD reflects a reduction in the quantal content of neurotransmitter release**

Analysis of miniature EPSCs (mEPSCs) was employed to examine whether LF-STD is presynaptic or postsynaptic and to further define the underlying cause of depression. An mEPSC is thought to represent the postsynaptic response to neurotransmitter release from a single vesicle (or quantum) and thus the mEPSC amplitude is a common measure of quantal size. In contrast, the EPSC evoked by a presynaptic action potential is produced by the nearly synchronous fusion of many vesicles. The number of vesicles contributing to the EPSC is referred to here as the quantal content. To explore the mechanism of LF-STD, the amplitude and frequency of mEPSCs recorded during a 60-s period before stimulation were compared with those of mEPSCs recorded during steady-state depression elicited by 1-Hz stimulation (Fig. 2). The mean mEPSC amplitudes were 0.71 ± 0.02 and 0.80 ± 0.06 nA, respectively (n = 5), indicating that no significant change in mEPSC amplitude was associated with depression. Similar mEPSC frequencies were observed prior to stimulation (1.06 ± 0.11/s) and during steady-state depression (0.93 ± 0.15/s). These results indicate that quantal size is unchanged during LF-STD and thus define the underlying mechanism as a reduction in the quantal content of evoked neurotransmitter release. A change in quantal content during depression may result from either a change in the size of the readily releasable pool or the release probability for vesicles within that pool.

**Degree of LF-STD is independent of the initial level of neurotransmitter release**

The progressive reduction in quantal content observed during LF-STD might result from depletion of a readily releasable pool of vesicles. In a depletion model, the level of depression should depend on the fraction of this pool released per stimulus (F), which is reflected in the initial level of neurotransmitter release (Zucker and Regehr 2002). To examine this possibility, solutions containing different extracellular Ca\textsuperscript{2+} concentrations expected to produce a range of F values were employed to adjust the level of neurotransmitter release evoked by calcium influx. The extent of depression was similar in solutions containing 0.5, 1.0, or 1.8 mM extracellular calcium for both 1- and 0.2-Hz stimulation, despite marked differences in the initial EPSC amplitude (Fig. 3). These findings indicate that the initial level of neurotransmitter release did not influence the degree of LF-STD and thus appear to be incompatible with a depletion model.

The relationship of the initial EPSC amplitude to the degree of LF-STD was also examined in two temperature-sensitive (TS) paralytic mutants, cac\textsuperscript{TS2} and shi\textsuperscript{TS1}, which exhibit reduced neurotransmitter release at elevated temperatures. cac\textsuperscript{TS2} is a temperature-sensitive paralytic allele of the presynaptic voltage-gated calcium channel \( \alpha 1 \) subunit gene, cacophony. Previous genetic and functional analysis of cac\textsuperscript{TS2} at adult neuromuscular synapses (Kawasaki et al. 2000, 2002), as well as imaging of cac-encoded \( \alpha 1 \) subunits at active zones...
by depleting synaptic vesicles and ultimately the readily releasable pool (Delgado et al. 2000; Estes et al. 1996; Kuromi and Kidokoro 1998; Li and Schwarz 1999; Macleod et al. 2004; Ramaswami et al. 1994; Verstreken et al. 2002). To examine the consequences of the shi<sup>TS1</sup> mutation for LF-STD, 1-Hz stimulation trains (15 pulses in duration) separated by 100-s rest intervals were delivered to wild type and shi<sup>TS1</sup> synapses at a restrictive temperature of 33°C. As expected, each stimulation train in wild type elicited LF-STD, which recovered during the inter-train interval (Fig. 5A). In shi<sup>TS1</sup>, the initial EPSC amplitude was similar to wild type for the first two stimulation trains and, consistent with previous studies, declined progressively thereafter (Fig. 5B). Comparison of the initial EPSC amplitudes for the first and eighth trains may serve as an example. In wild-type, the initial EPSC amplitude of the eighth train was sustained at $97.9 \pm 3.9\%$ ($n = 3$) relative to that of the first train. In contrast, the corresponding value in shi<sup>TS1</sup> was $72.3 \pm 2.3\%$ ($n = 4$). Despite this clear reduction in the initial EPSC amplitude in shi<sup>TS1</sup>, the degree of LF-STD elicited by the eighth train remained similar to wild type (Fig. 5C). Thus the degree of LF-STD persists in shi<sup>TS1</sup> at

\[ \text{FIG. 3. LF-STD persists in a wide range of extracellular calcium concentrations.} \]

\[ \text{A: the 1st and steady-state responses of a 1-Hz stimulation train were superimposed to represent initial and steady-state EPSCs in the different extracellular calcium concentrations indicated.} \]

\[ \text{B: comparison of initial EPSC amplitude and steady-state depression elicited by 1- or 0.2-Hz stimulation over a range of extracellular calcium concentrations. Initial EPSC amplitudes in 0.5, 1.0, and 1.8 mM calcium were $82.9 \pm 5.8$ ($n = 13$), $142.7 \pm 7.4$ ($n = 13$), and $230.5 \pm 18.7$ ($n = 14$). For 1-Hz stimulation, the respective EPSC<sub>C0</sub>/EPSC<sub>C0</sub> values in 0.5, 1.0, and 1.8 mM extracellular calcium were 0.71 $\pm$ 0.03 ($n = 5$), 0.72 $\pm$ 0.02 ($n = 5$), and 0.71 $\pm$ 0.03 ($n = 6$). The corresponding values for 0.2-Hz stimulation were 0.79 $\pm$ 0.02 ($n = 8$), 0.80 $\pm$ 0.02 ($n = 8$), and 0.75 $\pm$ 0.02 ($n = 9$). Depression was elicited by a 50-pulse stimulation train.} \]

\[ \text{shiTS1 is a TS paralytic allele of the dynamin gene, shibire.} \]

Previous work indicated that shi TS mutations disrupt synaptic vesicle recycling at larval neuromuscular synapses exposed to restrictive temperatures and thus produce a progressive activity-dependent reduction in neurotransmitter release. Unlike the preceding manipulations of calcium influx intended to modify $F$, the shi<sup>TS1</sup> mutation likely reduces neurotransmitter release.

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\[ \text{FIG. 4. LF-STD in a temperature-sensitive presynaptic calcium channel mutant, cacTS2.} \]

\[ \text{A: synaptic currents were recorded in wild-type (WT) and cac<sup>TS2</sup> larvae at 20 and 36°C. The 1st and steady-state responses to 1-Hz stimulation were superimposed.} \]

\[ \text{B: initial EPSC amplitudes and the degree of short-term synaptic depression elicited by 1-Hz stimulation for 100 s are shown in wild type and cac<sup>TS2</sup> at 36°C. Despite the reduction in initial EPSC amplitude, cac<sup>TS2</sup> exhibited the same degree of short-term depression observed in wild type. EPSC<sub>C0</sub>/EPSC<sub>C0</sub> values for wild type and cac<sup>TS2</sup> at 36°C were $0.63 \pm 0.02$ ($n = 9$) and $0.65 \pm 0.03$ ($n = 13$), respectively.} \]
restrictive temperature despite a progressive reduction in the initial EPSC amplitude over a series of stimulation trains. These results further confirm the lack of dependence of LF-STD on the initial level of neurotransmitter release. Given that prolonged stimulation in shiTS1 probably reduces the size of the releasable pool [cf. Li and Schwarz 1999], these results also indicate that LF-STD may be insensitive to the size of this pool.

Properties of LF-STD are independent of dynamin function

The results shown in Fig. 5 suggest that during the first stimulation trains, the characteristics of LF-STD are similar at shiTS1 and wild-type larval synapses at restrictive temperature. To further address this issue, the degree of LF-STD as well as the time course of its development and recovery, were examined for the initial 1-Hz stimulation trains at 33°C. A comparison of wild type and shiTS1 revealed no difference with respect to the degree and characteristics of LF-STD (Fig. 6), suggesting that the properties of LF-STD do not reflect dynamin function. Single-exponential curve fits to the time course of depression in wild type and shiTS1 yielded a τ value of 2.1 s in each case. Similar results were obtained in a second TS paralytic shibire mutant, shiTS2, in which the time course of depression exhibited a τ of 2.2 s. In contrast, the same shi mutations produce a striking synaptic depression phenotype at adult DLM synapses during the first 1-Hz stimulation train at 33°C, indicating a rapid activity-dependent role for dynamin at adult neuromuscular synapses (Kawasaki and Ordway 2000).

Two components of short-term depression at larval neuromuscular synapses

The preceding results reveal a substantial level of neurotransmitter release (>60%) that is not depressed at stimulation frequencies ≤20 Hz. However, increasing the stimulation frequency to 40 or 60 Hz depressed this residual release in a frequency-dependent manner (Fig. 7A and B). This second component of short-term depression may be analogous to depression observed at comparable stimulation frequencies in a variety of preparations, which often exhibits a strong dependence on frequency as well as the initial EPSC amplitude (Betz 1970; Dittman and Regehr 1998; Dobrunz and Stevens 1997; Kusano and Landau 1975; Mennerick and Zorumski 1995; Wang and Kaczmarek 1998; Zucker and Regehr 2002). Steady-state depression at 40 or 60 Hz, unlike that at lower frequencies, is dependent on the initial EPSC amplitude in different extracellular calcium concentrations and thus may depend on fractional release of the release-ready pool (Fig. 7C).
At extracellular calcium concentrations <1 mM, depression was not observed at 40 or 60 Hz due to prominent facilitation at these higher frequencies (not shown). Facilitation was also evident in normal extracellular calcium during the initial stages of high-frequency trains (40 or 60 Hz; Fig. 7A) as observed in a variety of other systems (Betz 1970; Dobrunz and Stevens 1997; Parker 1995; Richards et al. 2003). Thus the degree of steady-state depression at these frequencies is probably influenced by underlying facilitation.

The properties of short-term depression at larval neuromuscular synapses were further defined by examining recovery from depression. Recovery was assessed by delivering a 100-pulse stimulation train followed by a test pulse at different time intervals. The slow recovery associated with LF-STD persisted at all stimulation frequencies, however stimulation at 40 or 60 Hz also elicited a second component of recovery with rapid kinetics (Fig. 8A). Note that the amplitude and time course of the slow component after 40- or 60-Hz stimulation are similar to those observed for recovery of LF-STD elicited at low frequencies (Fig. 8, A and C). Single-exponential curve fits to the slow recovery component after 1-, 20-, 40-, and 60-Hz stimulation yielded respective $\tau$ values of 13.8, 14.9, 18.2, and 14.9 s. These results indicate that the mechanism responsible for LF-STD also produces one component of short-term depression at high stimulation frequencies.

With respect to the fast component of recovery after 40- or 60-Hz stimulation, the EPSC amplitude transiently increased to a value exceeding 67% of the initial amplitude (Fig. 8A) and then returned to $\sim$60% prior to significant progression of the slow component. This observation is consistent with the presence of underlying facilitation (or augmentation) during recovery from high-frequency stimulation as suggested previously (Wu and Borst 1999). In the case of larval neuromuscular synapses, a prominent peak resulting from transient facilitation during recovery may be more easily resolved because of the relatively slow time course of the slow recovery component (see DISCUSSION). The early recovery time points after 40- and 60-Hz stimulation were fit by single-exponential curves with respective $\tau$ values of 154.4 and 92.1 ms (Fig. 8B). This fast component may be analogous to similar recovery components observed in a variety of model systems including the Calyx of Held (Sakaba and Neher 2001; Wang and Kaczmarek 1998), climbing fiber to Purkinje cell synapses (Dittman and Regehr 1998), and cultured hippocampal neurons (Mennerick and Zorumski 1995).

FIG. 7. Short-term synaptic depression elicited by high-frequency stimulation at larval neuromuscular synapses. A: EPSC amplitudes are plotted as a function of stimulus number at frequencies of 20, 40, and 60 Hz. B: the degree of steady-state depression for the data in A is plotted vs. stimulation frequency. Respective EPSC$_{SS}$/EPSC$_{0}$ values for 20-, 40-, and 60-Hz stimulation were 0.62 $\pm$ 0.02 ($n = 8$), 0.49 $\pm$ 0.03 ($n = 5$), and 0.28 $\pm$ 0.01 ($n = 6$). C: the dependence of depression on extracellular calcium. For 40-Hz stimulation, EPSC$_{SS}$/EPSC$_{0}$ values for 1.0 and 1.8 mM calcium were 0.73 $\pm$ 0.01 ($n = 3$) and 0.49 $\pm$ 0.03 ($n = 5$), respectively. The corresponding values for 60-Hz stimulation were 0.47 $\pm$ 0.04 ($n = 3$) and 0.27 $\pm$ 0.02 ($n = 5$).

FIG. 8. Two components of recovery from short-term depression elicited by high-frequency stimulation. A: recovery from depression following 40- or 60-Hz stimulation exhibited fast and slow components. B: the fast components after stimulation at 40 or 60 Hz were fit with single-exponential curves exhibiting respective $\tau$ values of 154.4 and 92.1 ms. C: the slow component exhibited a similar time course after low- and high-frequency stimulation. Single-exponential curves fit to the slow recovery components after 1-, 20-, 40-, and 60-Hz stimulation yielded $\tau$ values of 13.8, 14.9, 18.2, and 14.9 s, respectively.
The preceding results indicate two components of depression at high stimulation frequencies including maximal LF-STD and a second, more conventional component of depression elicited at high stimulation frequencies. These findings further illustrate the robust properties of LF-STD over a wide frequency range, despite marked changes in the degree of depression associated with the second component. Thus manipulations of the extracellular calcium concentration and stimulation frequency that either enhance or suppress conventional short-term depression appear to have little effect on the process underlying LF-STD, suggesting that these components of depression involve different mechanisms.

**DISCUSSION**

Larval neuromuscular synapses of *Drosophila* were found to exhibit a distinct form of short-term synaptic depression, LF-STD, which is sensitive to stimulation frequency over a narrow range of low frequencies and independent of the initial level of neurotransmitter release. A second component of depression was elicited at higher frequencies and resembled short-term depression observed previously in a wide variety of systems. The two components of short-term depression appear to be independent and are associated with temporally distinct components of recovery after high-frequency stimulation. The present study is focused on the properties of LF-STD. We discuss the relationship of LF-STD to analogous depression observed in several other model systems as well as previous work in the *Drosophila* larva (Delgado et al. 2000) and consider possible underlying mechanisms.

**Features of LF-STD and relevance of depletion models**

Early studies of short-term synaptic depression suggested that the observed activity-dependent reduction in synaptic transmission resulted from depletion of a releasable pool of neurotransmitter (Betz 1970; Kusano and Landau 1975; Liley and North 1953; Thies 1965). Subsequent work has in some cases been consistent with modified depletion models (Dittman and Regehr 1998; Dobrunz and Stevens 1997; Richards et al. 2003; Sakaba and Neher 2001; Stevens and Wesseling 1998; von Gersdorff and Borst 2002; von Gersdorff and Matthews 1997); however, other studies have shown that the features of such models, notably a strong dependence of depression on the fraction (F) of the release-ready pool released per stimulus, cannot be reconciled with the observed properties of depression (Armitage and Siegelbaum 1998; Bellingham and Walmsley 1999; Brody and Yue 2000; Waldeck et al. 2000; Zucker and Jan 1977). Alternative mechanisms have been proposed (see following text); however, at present, the molecular bases of these phenomena, and short-term depression in general, remain poorly understood.

Striking examples of low-frequency depression that do not conform to a depletion model were observed in an early study of crayfish abdominal neuromuscular synapses (Zucker and Jan 1977) and more recently at *Aplysia* sensorimotor synapses in culture (Armitage and Siegelbaum 1998). In both cases, depression occurred at very low frequencies (<0.02 Hz) and was relatively insensitive to stimulation frequency over a moderate range. As in the present work, both groups altered the initial level of neurotransmitter release by adjusting F, and this had little or no effect on the degree of depression. Higher stimulation frequencies were not employed, and thus it remains uncertain whether these synapses also exhibit distinct components of depression at high frequencies. Direct comparison with the properties of depression reported here is precluded by major differences in the recording solutions and temperatures. However, the above findings indicate that features of low-frequency depression at crayfish neuromuscular and *Aplysia* sensorimotor synapses, like those at neuromuscular synapses of *Drosophila* larvae, appear to be incompatible with a depletion model.

It is also of interest to compare the present results with those obtained in an elegant mammalian model for studies of synaptic transmission, the Calyx of Held. At Calyx of Held synapses from 8- to 10-day-old rats, short-term depression occurs at low stimulation frequencies (≥0.2 Hz) and is relatively insensitive to frequency over a moderate range (2–10 Hz) (von Gersdorff et al. 1997). Thus under comparable recording conditions, some overlap was observed in the properties of low-frequency depression at the Calyx of Held and larval neuromuscular synapses of *Drosophila*. However, clear differences were also evident with respect to the dependence of depression on the initial EPSC amplitude as well as the time course of recovery. While depression at the Calyx of Held was not examined under conditions altering the initial level of neurotransmitter release, correlated variation in the degree of depression and the initial EPSC amplitude suggests depression is enhanced at higher levels of neurotransmitter release. With respect to recovery from depression, in fact Calyx of Held presynaptic terminals (von Gersdorff et al. 1997; Wang and Kaczmarek 1998) resemble those of rat hippocampal synapses in brain slices (Dobrunz and Stevens 1997) and cell culture (Brody and Yue 2000; Mennerick and Zorumski 1995; Stevens and Wesseling 1998), rat climbing fiber neurons (Dittman and Regehr 1998), frog motor neurons (Betz 1970), locust motor neurons (Parker 1995), and the squid giant synapse (Kusano and Landau 1975), all of which exhibit a prominent slow component of recovery with a τ of ~3–6 s. This recovery has often been modeled as refilling of the readily releasable pool. In contrast, LF-STD at larval neuromuscular synapses exhibits a somewhat slower recovery time course with a τ of ~15 s (Fig. 8). Given that the properties of LF-STD appear to be incompatible with a depletion model, recovery of this process is likely to reflect a mechanism other than, or in addition to, refilling of a releasable pool.

The properties of short-term synaptic depression at the Calyx of Held are not consistent with predictions of a simple depletion model; however, they may be explained by a modified depletion model involving a heterogenous releasable pool consisting of both rapidly and reluctantly releasable vesicles (Sakaba and Neher 2001; Trommershauser et al. 2003; von Gersdorff and Borst 2002; Wu and Borst 1999). Recovery from short-term depression elicited by high-frequency stimulation exhibits a rapid component (τ ≈ 100 ms) in addition to the slow component described in the preceding text (Sakaba and Neher 2001; Wang and Kaczmarek 1998; Wu and Borst 1999). This appears to reflect a conserved process in that two similar recovery components have been observed in other systems (Dittman and Regehr 1998; Mennerick and Zorumski 1995), including adult neuromuscular synapses of *Drosophila* (Kawasaki and Ordway, personal communication). Several studies
have reported that residual calcium contributes to the fast recovery component (Dittman and Regehr 1998; Sakaba and Neher 2001; Wang and Kaczmarek 1998) by facilitating release from a reluctant pool of vesicles (Wu and Borst 1999) and accelerating refilling of a rapidly releasing pool (Sakaba and Neher 2001). It is of interest to consider whether similar processes may contribute to rapid recovery from depression after high-frequency stimulation at larval neuromuscular synapses, which exhibits a comparable recovery time course ($\tau \approx 100$ ms).

Comparison with previous work at Drosophila neuromuscular synapses

A recent study at larval neuromuscular synapses of Drosophila defined distinct synaptic vesicle pools and their dynamics during repetitive stimulation (Delgado et al. 2000). These pools were inferred from curve fits to the decay of EPSCs during repetitive stimulation in the shiTS1 mutant, assuming that activity-dependent EPSC reduction may be attributed to depletion of vesicle pools. Recovery was not examined. Although the main focus of this work is distinct from that of the present study, it is of interest to compare common elements. Although the data in the two studies are generally consistent, an unexplained discrepancy arises with the previously reported dependence of steady-state depression on stimulation frequency over a moderate frequency range (Delgado et al. 2000). A more central issue involves the respective kinetics of depression in the two studies. The previous study demonstrated that the decay of EPSC amplitudes in shiTS1 mutants at restrictive temperature exhibited several kinetic components, including a fast component that showed no clear dependence on stimulation frequency. The fast component in shiTS1 exhibited a time constant (expressed in terms of stimulus number) of $\tau = 2.3$ stimuli for 10-Hz stimulation at 32°C. This value is quite similar to the time course observed for LF-STD in the present study ($\tau = 2.1$ stimuli for 1-Hz stimulation at 33°C; see legend to Fig. 6). The fast component was attributed to an immediately releasable vesicle pool. However, in contrast to intermediate and slow components of depression, the fast component was not observed in parallel imaging experiments monitoring vesicle depletion with the fluorescent dye, FM 1–43. Although this observation may reflect the small size of an immediately releasable pool (Delgado et al. 2000), it may also indicate that the fast component is produced by a mechanism other than depletion. The preceding observations suggest that the fast component of depression in the previous study may correspond to LF-STD.

Mechanism of LF-STD

Of the studies discussed in the preceding text, those at Calyx of Held and Aplysia sensorimotor synapses are particularly informative regarding the underlying mechanisms producing short-term depression at low stimulation frequencies (Armitage and Siegelbaum 1998; von Gersdorff and Borst 2002; von Gersdorff et al. 1997). These studies demonstrated that depression was presynaptic and employed imaging and electrophysiology methods to show that depression did not result from altered action potential invasion of presynaptic terminals or the resulting calcium influx. Consistent with these results, single bouton recordings from larval neuromuscular synapses of Drosophila indicate that individual boutons can follow paired-pulse nerve stimulation at an interpulse interval of 40 ms (Renger et al. 2000). The preceding observations indicate that at least some presynaptic determinants of short-term synaptic depression elicited by low-frequency stimulation are downstream of calcium entry. In addition to depletion of the readily releasable pool, a variety of other downstream mechanisms have been proposed to mediate short-term depression, including direct feedback of neurotransmitter on presynaptic terminals and activity-dependent adaptation/inactivation of the neurotransmitter release apparatus (Armitage and Siegelbaum 1998; Hsu et al. 1996; Redman and Silinsky 1994; von Gersdorff and Borst 2002; Zucker and Regehr 2002). Clearly additional analysis of short-term depression is required to further define the underlying molecular mechanisms.

In light of the sophisticated genetic approaches possible in Drosophila, as well as the widespread use of the larval neuromuscular synapse preparation for studies of synaptic transmission, the features of short-term depression defined here will provide an important context for further dissection of the in vivo molecular mechanisms governing short-term synaptic depression. The present study initiates this process by examining TS paralytic mutations in specific genes encoding a primary presynaptic calcium channel structural subunit as well as dynamin. The finding that the properties of LF-STD were not dependent on dynamin function is instructive, particularly in light of the rapid requirement for dynamin during repetitive stimulation of adult neuromuscular synapses (Kawasaki and Ordway 2000). These findings emphasize that some diversity exists in the molecular mechanisms of neurotransmitter release even among neuromuscular synapses of a single organism. Further genetic analysis of short-term synaptic depression in Drosophila is expected to advance our understanding of neurotransmitter release mechanisms and their dependence on activity.

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