Requirement of Akt to Mediate Long-Term Synaptic Depression in *Drosophila*

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Drosophila larval neuromuscular junction (NMJ) is a well established preparation enabling quantitative analyses of synaptic physiology at identifiable synapses. Here, we report the first characterization of synaptic long-term depression (LTD) at the *Drosophila* NMJ. LTD can be reliably induced by specific patterns of tetanic stimulation, and the level of LTD depends on both stimulus frequency and Ca^{2+} concentration. We provide evidence that LTD is likely a result of presynaptic changes. Through screening of targeted mutants with defects in memory or signal transduction pathways, we found that LTD is strongly reduced in the *akt* mutants. This defect can be rescued by acutely induced expression of the normal *akt* transgene, suggesting that altered LTD is not attributable to developmental abnormalities and that Akt is critical for the induction of LTD. Our study also indicates that the molecular mechanisms of LTD are distinct from that of short-term synaptic plasticity, because *akt* mutants showed normal short-term facilitation and posttetanic potentiation, whereas LTD was unaffected in mutants that exhibit defective short-term synaptic plasticity, such as *dunce* and *rutabaga*. The characterization of LTD allows genetic analysis of the molecular mechanisms of long-term synaptic plasticity in *Drosophila* and provides an additional assay for studying functions of genes pertaining to synaptic and behavioral plasticity.

Key words: long-term depression; neuromuscular junction; synaptic plasticity; *Drosophila*; Akt; short-term plasticity

Introduction

The larval neuromuscular junction (NMJ) is the only preparation in *Drosophila* suitable for quantitative analysis of synaptic transmission at identifiable synapses. It has been used extensively to study the molecular basis of synapse development, synaptic plasticity (for review, see Keshishian et al., 1996; Packard et al., 2003), synaptic vesicle release (for review, see Schwarz, 1994; Wu and Bellen, 1997), and functions of genes involved in learning and memory (Zhong and Wu, 1991; Broadie et al., 1997; Guo et al., 1997, 2000; Rohrbough et al., 1999, 2000; DeZazzo et al., 2000). Various forms of short-term synaptic plasticity at the NMJ have been demonstrated, including facilitation, augmentation, posttetanic potentiation, and depression (Jan and Jan, 1978; Zhong and Wu, 1991; Broadie et al., 1997; Delgado et al., 2000; Wu et al., 2005), with durations ranging from seconds to several minutes. These forms of plasticity are disrupted in a number of mutants with defective intracellular signal transduction pathways and impaired learning and memory (Zhong and Wu, 1991; Rohrbough et al., 1999, 2000), such as *dunce* and *rutabaga* that express mutated forms of cAMP-specific phosphodiesterase and adenylyl cyclase, respectively (Chen et al., 1986; Levin et al., 1992). However, neither long-term potentiation nor long-term depression

(LTD) has been demonstrated at the glutamatergic synapses of the NMJ. If one of these forms of long-lasting plasticity could be demonstrated at these synapses, it would be possible to use *Drosophila* genetic tools to analyze the molecular mechanisms of long-term synaptic plasticity.

Accumulating data suggest that the molecular mechanisms for long-term synaptic plasticity exist also at the *Drosophila* NMJ. Genetic manipulations of these molecules were shown to produce long-term modifications of synaptic strength. For example, synaptic transmission at the NMJ was persistently enhanced in mutants of *dunce* as a result of elevated levels of cAMP (Zhong and Wu, 1991; Renger et al., 2000) and was reversed by inhibiting the activity of the transcription factor cAMP response element-binding protein (CREB) (Davis et al., 1998). Genetic manipulations of local protein synthesis, glutamate receptors expression (Sigrist et al., 2000, 2002, 2003), and the activator protein-1 transcription factors (Sanyal et al., 2002) were also shown to modify the synaptic strength at these synapses. Thus, various signaling systems underlying long-term plasticity in vertebrates have also been observed in the synapses of *Drosophila* NMJ.

Long-term plasticity has been reported at the NMJ of both vertebrates and other invertebrates (Lnenicka and Atwood, 1985; Lo et al., 1994; Cash et al., 1996; Malenka and Nicoll, 1999; Wan and Poo, 1999; Etherington and Everett, 2004). We therefore attempted to induce similar long-term plasticity at the *Drosophila* larval NMJ. Here we show that LTD can be reliably induced after the delivery of a specific pattern of electrical stimulation to the motor axons. In the present study, we characterized the properties of this newly identified LTD and examined LTD in selected mutant flies.

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Materials and Methods

Fly care and heat shock treatment. All flies were raised at room temperature (RT) in regular cornmeal food (unless otherwise indicated). To induce the expression of *hsp70-akt* (normal *akt* transgene driven by the promoter of heat shock protein 70) in the *hsp70-akt; akt¹* flies, heat shock (HS) treatment (30 min at 37°C water bath) was delivered once a day starting from the embryonic stage (developmental daily HS treatment). This HS treatment to *hsp70-akt; akt¹* was sufficient to overcome the lethality of the homozygous *akt¹* mutant allele and provided viable third-instar larvae for electrophysiological analysis. Specifically, flies were allowed to lay eggs for 1 d in the bottle and then were removed from the bottle. HS treatment began after removing the flies. After the last daily heat shock exposure, the *hsp70-akt; akt¹* larvae were placed at 18°C for 24–48 h to reduce the leaky expression of the *hsp70-akt* transgene. Some of these larvae were brought to RT for 3–6 h and were then subjected to HS treatment (37°C, two exposures of 15 min, 2 h interval) (see Fig. 7A, 18°C→HS). Dissection of larvae and recordings for the 18°C→HS group was performed at 0.5–2 h after the second 15 min HS exposure. For control, some larvae were brought to RT from 18°C and directly dissected for electrophysiological recordings at RT (see Fig. 7A, 18°C). Some larvae were not placed at 18°C but were subjected to dissection and recordings at 24 h after the last daily HS treatment (see Fig. 7C, RT group) or were treated by the same HS paradigm as for 18°C→HS at 24 h after the daily HS treatment and then subjected to electrophysiological analysis.

Immunohistochemistry and measurement of fluorescence intensity. Immunostaining of *Drosophila* Akt (dAkt) (1:200) on the larval NMJ was performed according to the method described previously (Rohrbough et al., 2000) using a polyclonal Akt antibody. The secondary antibody was FITC conjugated (1:1000). Fluorescence color images were taken by confocal laser scanning microscopy. For measurement of the staining intensity and for clearer presentation of the staining effect, the fluorescence images were inverted using Scion NIH Image (Scion, Frederick, MD) (see Fig. 9).

Electrophysiology. Electrophysiological recordings of two-electrode voltage clamp were performed as described previously (Stewart et al., 1994; Zhong and Pena, 1995). For optimal long-term recording, wall-climbing third-instar larvae from large fresh bottles (without adult flies in the bottle) were chosen for dissection. Dissections of third-instar larvae were made at RT and in Ca^{2+} -free hemolymph-like (HL-3) solution (Stewart et al., 1994; modified by Feng et al., 2004) containing the following (in mM): 70 NaCl, 5 KCl, 4 MgCl_2 , 10 NaHCO_3 , 5 trehalose, 5 HEPES, and 115 sucrose. For recordings, HL-3 solution was supplemented with CaCl_2 (concentrations are indicated in the text and the figure legends). All recordings were made at the longitudinal muscles of segments A3–A5. To elicit evoked junctional currents (EJCs), the segmental nerve was stimulated at 1.5 times the stimulus voltage required for a threshold response, unless otherwise indicated. For recordings of LTD and controls, continuous recordings were made while the nerve was stimulated at baseline frequency of 0.05 Hz. For induction of LTD, tetanus of defined frequency and duration (see figure legends) was delivered after ~5 min of baseline stimulation. Methods for induction of short-

Figure 1. LTD at the *Drosophila* NMJ. **A**, Representative recordings of control and LTD induction. EJCs were recorded at the NMJ while the segmental nerves innervating the corresponding muscle fibers were stimulated at the baseline frequency of 0.05 Hz. For induction of LTD, tetanus of 30 Hz (20 s) was delivered at ~5 min after continuous recording. Calibration: 20 nA, 2 min. **B**, Summary of normalized EJC amplitude in control ($n = 6$) and LTD ($n = 13$) recordings. Time of -5 min refers to the time before the tetanus, which applies to all figures. Arrow indicates delivery of tetanus. **C**, Comparison of quantal content in the control and LTD recordings. Note that, in contrast to EJCs, the quantal content in the control remained stable within 35 min. **D**, Dependency of LTD on tetanus frequency (5–50 Hz, 20 s; 0.4 mM Ca^{2+}). $n = 4, 4, 4, 4, 4, 4, 4, 4$ for the tetanus frequency of 0, 5, 10, 20, 30, 40, 50 Hz, respectively. **E**, Dependency of LTD on Ca^{2+} concentrations (0.2–1.0 mM, 30 Hz for 20 s). $n = 4, 4, 4, 4, 4, 4, 4, 4$ for no tetanus and 0, 0.2, 0.4, 0.7, 1.0 mM Ca^{2+} , respectively. For Figures 1, 2, and 4–7, all recordings were performed on M12 in segment 3–5 and at 0.35–0.4 mM Ca^{2+} .

term facilitation (STF) and posttetanic potentiation are detailed in the figure legends. Current signals were amplified with an Axoclamp 2A amplifier (Molecular Devices, Palo Alto, CA). The signals were filtered at 0.1 kHz on-line and converted to a digital signal using a Digidata 1320A interface (Molecular Devices), acquired by pClamp 8.0 software (Molecular Devices). Stimulation of nerves was achieved by a Grass Instruments (Quincy, MA) S88 Stimulator. Pressure injection of glutamate (100 mM) was performed using Picospritzer II (General Valve, Fairfield, NJ).

Data analyses and statistics. Evoked and spontaneous responses were analyzed using the Mini Analysis Program (Synaptosoft, Decatur, GA). For continuous long-term recordings, amplitude of each EJC was normalized to the average EJC amplitude before the tetanus. Each time point represents average from three (before tetanus) or six (after tetanus) consecutive EJCs. For analysis of miniature EJCs (mEJCs), continuous recordings of 1 min (the first 1 min) in each 5 min period were taken for analysis. Quantal amplitude (quantal size) was determined using either the “Gaussian” (for a single peak) or the “10 Simplex” fitting functions in the Origin program (Microcal, Southampton, MA). For each recording, values of average mEJC amplitude and quantal content were normalized to that before tetanus. Quantal content are calculated as dividing average EJC amplitudes by quantal amplitude. Other details of data analysis are described in the figure legends.

Results

Induction of LTD

EJCs were recorded from the longitudinal muscle fiber 12 (M12) (for nomenclature, see Johansen et al., 1989a,b; Vactor et al., 1993), which has been examined extensively in physiological studies (Zhong and Wu, 1991; Davis et al., 1998; Rohrbough et al., 1999, 2000; Sigrist et al., 2000). Continuous recordings were made while the segmental nerves innervating the corresponding muscle cells were stimulated using a baseline stimulation frequency of 0.05 Hz. We sought to induce long-term synaptic plasticity by delivering various patterns of tetanic stimulation after ~5 min of baseline stimulation. LTD was consistently induced by 30 Hz tetanus for 20 s at 0.4 mM external Ca^{2+} concentration (Fig. 1A–C). It is important to monitor synaptic responses for synaptic failures during the tetanic stimulation that might occur in some preparations, which would then lead to attenuated LTD. In this study, muscle fibers with failures of evoked responses during tetanus (~20%) were not included for analysis. We have been able to maintain stable recording for a maximum of 60 min after LTD induction; LTD persisted throughout the recording period. However, some preparations became unstable after 45 min of recordings, i.e., leakage currents were increased dramatically. We therefore only present data herein recorded within 30 min after tetanus.

In muscle fibers not subjected to high-frequency stimulation, the EJC amplitude also decayed slightly after a long period of recordings (Fig. 1A,B) but to a much lesser extent than that after LTD induction. The most likely explanation for this decay is a reduction in the quantal amplitude [quantal size (presented later in Fig. 4A,C)], which is consistent with a previous report of the reduced quantal size after extended period of recording at the *Drosophila* NMJ (Davis et al., 1998). After accounting for the reduced quantal size, we determined that the quantal contents (dividing EJC amplitude by quantal amplitude), in fact, were not changed significantly during the course of recording in the controls (Fig. 1C).

Stimulus frequency and Ca^{2+} concentration dependence of LTD

The frequency of tetanic stimulation is critical for induction of LTD. We examined a series of stimulation frequencies, each with duration of 20 s. LTD was not observed after stimulation at 5 or 10 Hz but was induced after 20 Hz stimulation. The level of LTD reached a plateau at 30 Hz, and similar levels of LTD were observed at higher frequencies, such as 40 or 50 Hz (Fig. 1D). We also examined 10 Hz stimulation for 60 s, which delivers an equal number of stimuli as does 30 Hz stimulation for 20 s. This prolonged 10 Hz stimulation, however, did not elicit LTD (data not shown). Thus, it is the stimulation frequency rather than the total number of stimulation that is crucial for induction of LTD.

External Ca^{2+} concentration is another critical factor for LTD induction. In the absence of external Ca^{2+} (the saline was replaced with Ca^{2+} -free saline during the tetanic stimulation), 30 Hz stimulation failed to elicit LTD (Fig. 1E). At the range of 0.2–1 mM $[\text{Ca}^{2+}]$, however, the level of LTD was inversely related to the Ca^{2+} concentrations (Fig. 1E). LTD was most pronounced at 0.2 mM Ca^{2+} , lesser at 0.4 mM, and further reduced at 0.7 mM. At 1 mM, only a shorter form of depression (lasting for ~10 min) was observed. These data suggest that relatively low levels of Ca^{2+} are essential for induction of LTD, whereas higher levels appear to impede LTD induction.

Next we examined whether muscle contraction would disrupt

Figure 2. An experiment indicating normal NMJ function after the induction of LTD. Tetanus of 15 Hz (15 s) was delivered at 20 min after LTD induction (30 Hz, 20 s); it induced short-term facilitation, posttetanic potentiation, and partial reversal of LTD. **A**, Representative traces. Calibration: 20 nA, 2 min. **B**, Summary ($n = 5$). Arrows indicate the delivery of 30 and 15 Hz tetanus.

the functions of the synaptic terminals, which might mimic LTD. To test the functions of the NMJ after the induction of LTD, we delivered 15 Hz (15 s) stimulation to the depressed nerve terminals at 20 min after the 30 Hz stimulation. The NMJ was able to respond to this novel tetanus without failure of transmission and exhibited STF and posttetanic potentiation like the normal NMJ. Moreover, the depression was partially reversed by the 15 Hz stimulation (Fig. 2). These results suggest that muscle contraction resulting from the induction of LTD did not disrupt NMJ function.

Induction of LTD at different NMJs

We investigated whether LTD can be induced in muscle fibers other than M12. In each hemisegment, there are 30 individual muscle fibers, each being innervated by multiple motor nerve terminals (Johansen et al., 1989a,b; Kurdyak et al., 1994; Keshishian et al., 1996; Lnenicka and Keshishian, 2000). In addition to M12, we examined M4 and M6, which have also been frequently studied. LTD was also observed at M4 and M6 (Fig. 3A,B), but the dynamics of depression were different. There appear to be two components of depression in M12, consisting of LTD and a short-term depression (STD) that lasts for ~10 min. In contrast, this short-term depression is absent from M4 and M6, although similar levels of LTD were observed at these fibers. These data suggest that LTD can be

Figure 3. Induction of LTD at various longitudinal muscle fibers M4, M6, and M12 (**A, B**) and at different axons (**C–E**). **A**, Representative traces. Calibration: 20 nA, 2 min. **B**, Summary of normalized EJC amplitude. $n = 4, 6, 4$ for M4, M6, M12, respectively. **C**, A representative recording on M6 by retrograde stimulation of axon 2 on the nerve branch to M12. Calibration: 10 nA, 2 min. **D**, Summary of experiments on stimulating axon 2. $n = 4$. **E**, Comparison of the responses on M6 evoked by stimulating axon 2 and the total EJC evoked by stimulating the segmental nerve. Note that the total EJC amplitudes of the segmental nerve were larger than those of axon 2 after induction of LTD, suggesting that both axons were responsive after LTD induction. $n = 4$ for either group.

induced at different muscle fibers, although with different dynamics.

Because each muscle fiber is innervated by multiple axons, we asked whether the high-frequency stimulation caused failure of synaptic transmission in individual axons innervating the same muscle fiber. If this were the case, the observed LTD-like phenomenon would be a result of an inability to generate action potentials from one or more axons rather than depression of synaptic transmission. To investigate this possibility, we took advantage of different innervating patterns of M6 and M12: M6 is innervated by axons 1 and 2, whereas M12 is innervated by axon 2 but not axon 1 (Kurdyak et al., 1994; Lnenicka and Keshishian, 2000). Stimulating the nerve branch innervating M12 allows retrograde stimulation of only axon 2 and recording of its corresponding synaptic responses (EJCs) at M6. The corresponding EJCs were small and highly stable, without depression after the 30 Hz stimulation (Fig. 3C,D). In contrast, the total EJCs in response to stimulation of the segmental nerve (including both axons 1 and 2) were depressed after the 30 Hz stimulation but were significantly larger than EJCs evoked by stimulating axon 2 alone (Fig. 3E). Thus, both axon 1 and axon 2 were responsive after the induction of LTD. These observations suggest that LTD did not result from a failure to generate action potentials from either axon.

Presynaptic mechanism

We then analyzed whether LTD was of presynaptic or postsynaptic origin. First, we performed quantal analysis. In the control without delivery of tetanic stimulation, the quantal amplitude (quantal size, usually representing postsynaptic properties) de-

creased gradually with time (Fig. 4A,C). This decrease accounts for the gradual decay in EJC amplitude in the control (Fig. 1). After LTD induction, the quantal amplitude remained very similar to that before the induction of LTD (Fig. 4B,D), whereas the quantal content (reflecting the number of vesicles in each EJC) decreased correspondingly to the depression of EJCs (Fig. 1C). The frequency of mEJCs was significantly increased after LTD induction compared with the control (Fig. 4E), also suggesting there were presynaptic changes. Second, we determined whether the tetanic stimulation altered the muscle responses to exogenously applied glutamate, which is independent from transmitter release from the presynaptic compartment and therefore reflects only changes in postsynaptic properties. The currents induced by the locally perfused glutamate were not significantly different before and after LTD induction (Fig. 4F). Together, both the results of quantal analysis and the experiment of exogenous glutamate application indicate that LTD is likely a result of presynaptic changes (e.g., a reduced number of vesicles in each evoked response) rather than postsynaptic changes (e.g., the number or sensitivity of the glutamate receptors).

Disruption of LTD in *akt* mutants

Subsequent to the identification of LTD in normal flies, we conducted a specific genetic analysis to investigate the underlying molecular mechanisms. It is well known that signal transduction pathways are essential for synaptic plasticity, and synaptic plasticity is closely related to behavioral plasticity. We therefore examined LTD in a variety of mutants that exhibit impaired learning, abnormal synaptic function, or dysregulated signal transduction, such as *rutabaga*, *dunce* (Byers et al., 1981; Zhong and Wu, 1991; Levin et al., 1992), *latheo* (Rohrbough et al., 1999), *notch* (Ge et al., 2004; Presente et al., 2004; Costa et al., 2005), *gap1* [expressing a mutated Ras-specific GTPase-activating protein (Gaul et al., 1992)], and *akt* (with mutations in the gene encoding the protein kinase B/Akt).

LTD was not significantly affected in most of these mutants, including those that disrupt synaptic transmission or short-term synaptic plasticity (e.g., *rutabaga*, *dunce*, and *latheo*) (Fig. 5B). In contrast, LTD was strongly impaired in the viable *akt* mutant alleles *akt⁴²²⁶* and *akt⁴²²⁶/akt¹* (Fig. 5A). The hypomorphic allele *akt⁴²²⁶* harbors a P-element insertion upstream of the *dakt* gene and was reported to cause reduced expression of *akt* (Spradling et al., 1999; Gao et al., 2000; Stocker et al., 2002), whereas *akt¹* is an ethylmethyl sulfonate-induced null allele (embryonic lethal) because of a point mutation, dAktF327I, that confers a catalytically inactive kinase (Staveley et al., 1998; Stocker et al., 2002).

We next asked whether the impaired LTD in the *akt* mutant is attributable to altered dependency on Ca^{2+} concentration and tetanus frequency. Because LTD was most pronounced at 0.2 mM Ca^{2+} , we examined LTD in *akt⁴²²⁶* using this concentration. LTD

was similarly disrupted in the *akt* mutant in 0.2 mM Ca^{2+} saline as in 0.4 mM Ca^{2+} saline (Fig. 6*A,B*). We also tested whether higher-frequency stimulation (40 Hz) would overcome the deficit in LTD in the *akt* mutant; however, this was not the case either (Fig. 6*C,D*). These data suggest that impaired LTD in the *akt* mutant is not a result of altered dependency on Ca^{2+} concentration or stimulation frequency.

LTD was similarly disrupted in the trans-heterozygous allele *akt⁴²²⁶/akt¹* as in *akt⁴²²⁶*. However, *akt¹* is a null allele whereas *akt⁴²²⁶* is hypomorphic, so the trans-heterozygous allele should have less residual *akt* expression or activity than *akt⁴²²⁶*. We therefore examined LTD in the heterozygous *akt¹/+* to test the dose requirement of Akt for LTD. LTD in *akt¹/+* was not significantly different from that in the wild type (data not shown), suggesting that LTD is normal when the level of Akt is reduced by half. The *akt⁴²²⁶* mutant allele is semilethal (only a few homozygous flies survive), homozygous female sterile, and has smaller body size; these phenotypes are also not present in *akt¹/+*. These observations suggest that *akt⁴²²⁶* is a strong *akt* mutant allele. Thus, *akt⁴²²⁶* and *akt⁴²²⁶/akt¹* may possess similar levels of residual Akt expression or activity and therefore confer similar disruption of LTD. However, it is also possible that the reduction of *akt* expression in *akt⁴²²⁶* is sufficient to produce maximum disruption of LTD.

Rescue of LTD in *akt* mutants by induced expression of *akt* transgene

The impaired LTD in the *akt* mutants may be because that Akt is directly required to mediate LTD or because of developmental abnormalities. To distinguish between these possibilities, we tested whether the impaired LTD in *akt* mutants can be rescued by acutely induced expression of a normal *akt* transgene driven by the promoter of heat shock protein 70 (*hsp70-akt*) (Scanga et al., 2000). The *akt¹* mutant carrying *hsp70-akt* (*hsp70-akt;akt¹*) is a lethal allele and is therefore maintained over the third multiply inverted *TM6* balancer chromosome (with the marker *tubby*). However, the lethality can be overcome by daily HS treatment (37°C, 30 min; see Materials and Methods) to induce the expression of *hsp70-akt* from embryonic stage (Scanga et al., 2000). Thus, we were able to examine LTD in the larvae of *hsp70-akt;akt¹*.

To examine *hsp70-akt;akt¹* as an *akt* mutant for control purpose, it is necessary to silence or minimize the expression of the *hsp70-akt* transgene. The allele *hsp70-akt;akt¹* would be a null *akt* mutant allele if the expression of transgene could be completely silenced. There are several sources of leaky or residual expression of the *akt* transgene. First, there may be residual expression after the daily heat shock exposure. Sec-

Figure 4. Analysis of mEJCs and local glutamate perfusion-induced currents. Changes of mEJCs in the control (*A, C, E*) and before and after LTD induction (*B, D, E*). *A, B*, Representative mEJC traces. *C, D*, Histogram and Gaussian fitting of mEJCs. *E*, Summary of mEJC frequency changes. Quantal size and mEJC frequency were gradually reduced in the control (*A, C, E*). After LTD induction (15 min), quantal size remained unchanged, but there was an increased number of larger events that form a second peak, suggesting two quantal releases (*D*). As a result, the total frequency of mEJC also increased after the tetanus compared with the control (*E*). Calibration: 1 nA, 0.5 s. *n* = 8 for either group. *F*, Locally applied glutamate (100 mM)-induced currents ("G") did not significantly change after the induction of LTD. Top, Representative traces. Arrowheads indicate the glutamate-induced currents ("G"), which have slow kinetics; all other fast spikes are EJCs ("E"). Calibration: 20 nA, 1 min. Expanded traces before and after the 30 Hz tetanus are shown in the boxes (scale bars, 100 ms). Bottom, Summary. *n* = 6.

ond, the *hsp70* promoter may be leaky at room temperature. Third, we observed a few first-instar larvae homozygous for *akt¹* in *hsp70-akt;akt¹* even when the animals were raised at 18°C and were not exposed to heat shock, suggesting that there is a small amount of leaky expression of *hsp70-akt*. Apparently, such leaky expression is unrelated to the *hsp70* promoter, but is possibly attributable to local genomic enhancer(s) or promoter(s), and therefore cannot be eliminated by temperature adjustment. This small leaky expression prevented us from examining a null *akt* mutant allele. To minimize the leaky or residual *hsp70-akt* expression as a result of the *hsp70* promoter, we shifted the larvae homozygous for *akt¹* in *hsp70-akt¹;akt¹* (rescued by daily heat shock treatment) from RT to 18°C for 24–36 h after the last daily heat shock exposure.

We then tested whether impaired LTD in *akt* mutants can be

Figure 5. LTD was primarily reduced in the *akt* mutant alleles *akt⁴²²⁶* and *akt⁴²²⁶/akt¹* (**A**) but not affected in the mutants of *rutabaga*, *dunce*, and *latheo* (**B**). **A**, Representative recordings (top) (calibration: 20 nA, 2 min) and summary of normalized EJC amplitude (bottom) in control and *akt* mutant alleles. WT, Wild type. **B**, Representative LTD recordings in the mutants of *rutabaga* (*rut¹*), *dunce* (*dunce¹*), and *latheo* (*latheo^{P1}*).

rescued by acutely induced expression of *hsp70-akt*. The *hsp70-akt1;akt¹* larvae (homozygous for *akt¹*) from 18°C were examined for LTD either directly or after additional heat shock treatment (for heat shock paradigm, see below and Materials and Methods). Without additional heat shock, these larvae exhibited impaired LTD as did the larvae of the other *akt* mutants (Fig. 7A, 18°C), suggesting that *hsp70-akt1;akt¹* (homozygous for *akt¹*; 18°C) is an *akt* mutant allele and that shifting to 18°C effectively reduced the leaky expression of *hsp70-akt*. After additional heat shock treatment (37°C, two times for 15 min, 2 h interval; see Materials and

Methods), the impaired LTD in *hsp70-akt1;akt¹* larvae (18°C) was fully rescued (Fig. 7A, 18°C→HS). As a control, the same heat shock paradigm did not produce any effect on *akt⁴²²⁶* (data not shown), suggesting that the rescue effect is specifically attributable to *hsp70-akt* expression. These results indicate that acutely induced *hsp70-akt* expression was able to rescue the defective LTD in *akt¹* mutant.

We then examined whether impaired LTD in *akt⁴²²⁶/akt¹* can also be rescued by transiently induced expression of *hsp70-akt*. We crossed the *hsp70-akt;akt¹* with *akt⁴²²⁶*; therefore, there is one copy of *hsp70-akt* transgene in this allele. These larvae were viable at room temperature without heat shock treatment and showed impaired LTD like the other mutant alleles (Fig. 7B). Again, the impaired LTD in this allele was rescued by heat shock treatment (37°C, two to four times for 15 min, 1.5–2 h interval) (Fig. 7B). Thus, transiently induced expression of *hsp70-akt* was able to rescue impaired LTD in two *akt* mutant alleles, indicating that Akt is directly required to mediate LTD.

The degree of LTD rescue appears to be proportional to the amount of *hsp70-akt* expression. The *hsp70-akt;akt¹* larvae at RT exhibited partial rescue of LTD (examined at 24 h after the last daily HS), although with considerable variance, whereas they showed complete rescue shortly after additional heat shock treatment (two times for 15 min, 37°C, 2 h interval) (Fig. 7C). Similarly, the impaired LTD in *hsp70-akt; akt⁴²²⁶/akt¹* larvae was partially rescued after two exposures of heat shock (15 min, 37°C, 2 h interval) but fully rescued after four exposures of heat shock (15 min, 37°C, 1.5–2 h interval; $p < 0.01$) (Fig. 7D). These data further underscore the essential role of Akt for LTD.

Normal synaptic transmission and short-term synaptic plasticity in *akt* mutants

Drosophila NMJ exhibits multiple forms of short-term plasticity (Jan and Jan, 1978; Zhong and Wu, 1991; Broadie et al., 1997). To determine whether the impaired LTD in the *akt* mutants is a result of defective synaptic transmission or abnormal short-term synaptic plasticity, we examined spontaneous and evoked synaptic transmission, short-term facilitation, and posttetanic potentiation in the *akt* mutants. The amplitude and frequency of mEJC (Fig. 8A,B) and the amplitude and calcium dependency of EJCs (Fig. 8C,D) in *akt⁴²²⁶* and *akt⁴²²⁶/akt¹* are indistinguishable from those in the wild type. Similarly, short-term facilitation within the pulse train (Fig. 8E), the frequency dependence of short-term facilitation (Fig. 8F), and posttetanic potentiation (Fig. 8G) were also normal in the *akt* mutants. These data suggest that Akt is not essential for basic synaptic transmission and short-term plasticity.

Akt expression at the NMJ

We examined whether Akt is expressed at the *Drosophila* NMJ. We stained the NMJs of third-instar larvae using a polyclonal antibody against *Drosophila* Akt (Staveley et al., 1998) and observed strong dAkt-like immunoreactivity at the NMJ (Fig. 9). Consistent with previous studies (Spradling et al., 1999; Gao et al., 2000; Stocker et al., 2002), the dAkt staining is reduced in *akt⁴²²⁶*, as shown in both the synaptic boutons and the nerve branches (Fig. 9). The staining intensity measured in the synaptic boutons of *akt⁴²²⁶* was also significantly reduced compared with the wild type (wild type, 114.8 ± 3.1 ; *akt⁴²²⁶*, 73.1 ± 3.1 ; $p < 0.01$). These results indicate that Akt is expressed at the *Drosophila* NMJ.

Discussion

In the current work, we explored conditions for inducing LTD at the *Drosophila* larval NMJ and characterized the properties of LTD. LTD is dependent on the stimulation frequency and Ca^{2+} concentration and can be induced in various muscle fibers that are differentially innervated. Several observations indicate that the depression depends on synaptic transmission but not muscle contraction. First, after LTD induction, the NMJ responded normally to high-frequency stimulation and showed normal short-term synaptic plasticity, such as short-term facilitation and posttetanic potentiation (Fig. 2), and partial reversal of LTD from the novel stimulation (15 Hz). Second, LTD was attenuated at increased external Ca^{2+} concentrations when muscle contraction was more severe. Furthermore, LTD was disrupted in the *akt* mutants and rescued by acutely induced expression of the normal *akt* transgene.

The properties of LTD described here are similar to LTD reported previously in different preparations. For example, a major feature for LTD is the requirement of the presence of external Ca^{2+} , but its formation is also prevented if intracellular accumulation of Ca^{2+} is too high (Ito, 1989; Gall et al., 2005). Similarly, LTD at the *Drosophila* NMJ failed in the absence of Ca^{2+} but was most pronounced at relatively low (0.2 mM) external Ca^{2+} and attenuated with increasing Ca^{2+} concentrations. In other synaptic preparations, a long period of low-frequency stimulation (e.g., 1 Hz for 15 min) was typically used to induce LTD, albeit at high external Ca^{2+} concentrations. Although we used relatively high frequency stimulation (30 Hz), the relatively low external Ca^{2+} concentrations would limit Ca^{2+} influx; thus, similar internal Ca^{2+} concentrations may have been achieved in LTD induction at the *Drosophila* NMJ and other synapses. Our finding of a requirement of Akt for LTD also agrees with the report that the phosphatidylinositol 3 (PI-3) kinase/Akt/target of rapamycin (TOR) signaling is required for LTD in the hippocampus (Hou and Klann, 2004), although the role of Akt was not directly examined.

LTD in mammals can be divided into NMDA and non-NMDA receptor dependent and can be expressed at either the presynaptic site via a reduction in release probability or the postsynaptic site involving a decrease in AMPA receptor via clathrin-mediated endocytosis (Anwyl, 2006). It remains to be determined whether *Drosophila* LTD is NMDA or non-NMDA receptor dependent. However, our analyses indicate that *Drosophila* LTD is mainly expressed at the presynaptic site; therefore, it should not involve regulation of the number of postsynaptic AMPA receptors.

Short-term depression and LTD

STD at the *Drosophila* NMJ occurs during (but not after) high-frequency stimulation (10 Hz or higher) (Zhong and Wu, 1991; Delgado et al., 2000; Renger et al., 2000). Recently, a new type of STD during low-frequency stimulation of 0.5–1 Hz was reported (Wu et al., 2005). These forms of STD recover soon after termination of the stimulation. We observed similar STD

Figure 6. The *akt* mutant (*akt4226*) exhibited similarly impaired LTD at lower Ca^{2+} concentration (0.2 mM Ca^{2+} , at which LTD was the most pronounced in wild-type larvae) or by increased stimulation frequency (40 Hz). **A, B**, Reduction of LTD in the *akt* mutant at 0.2 mM Ca^{2+} was similar to LTD at 0.4 mM Ca^{2+} (refer to Fig. 1E). **A**, Representative recordings for wild type (WT) and *akt4226*. Calibration: 20 nA, 2 min. **B**, Summary of four recordings. **C, D**, LTD induced by 40 Hz tetanus was also primarily impaired in the *akt* mutant. **C**, Representative traces. Calibration: 20 nA, 2 min. **D**, Summary of four recordings.

during the high-frequency stimulation that induces LTD, which was not significantly different between the *akt* mutants and the wild type (data not shown). In addition, we observed a novel form of short-term depression that lasts for 10–15 min after the high-frequency stimulation at M12 but not M4 and M6 (Fig. 1). It is distinct from LTD because it was only elicited by 30 Hz or higher frequency stimulation but not by 20 Hz stimulation, which induced similar LTD, and was induced even at 1 mM Ca^{2+} when LTD was nearly absent (Fig. 1E). This short-term depression was also little affected in the *akt* mutants, in contrast to the disruption of LTD (Fig. 5). Thus, our data indicate that all forms of short-term plasticity are normal in *akt* mutants. In contrast, the mutants (e.g., *dunce*, *rutabaga*, and *latheo*) that exhibit defective short-term synaptic plasticity displayed normal LTD (Fig. 5B). These observations suggest that the mechanisms of LTD are distinct from those of STD and other forms of short-term synaptic plasticity.

It is believed that depletion of the readily releasable vesicle pool (RRP) is a candidate mechanism for short-term depression (Zucker and Regehr, 2002). A similar RRP and a reserved vesicle pool (RP) have been demonstrated at the *Drosophila* NMJ (Kuromi and Kidokoro, 2000; Kidokoro et al., 2004). However, two observations suggest that depletion of RRP did not occur after LTD induction. First, LTD was less pronounced at higher Ca^{2+} concentration (e.g., 1.0 mM), at which much more transmitter would have been released. Second, mEJC frequency should be decreased by depletion of RRP (Koenig and Ikeda, 1999; Delgado et al., 2000; Zucker and Regehr, 2002), but it was instead increased after LTD induction.

mEJC frequency and LTD

Along with LTD, there was increased mEJC frequency, which also appears to be long lasting (Fig. 4E). The mechanisms of mEJC frequency increase, and its relationship to LTD is not clear. One

Figure 7. Rescue of LTD in *akt¹* and *akt⁴²²⁶/akt¹* by acutely induced expression of *hsp70-akt* transgene. The *hsp70-akt;akt¹* embryos and larvae were given developmental daily heat shock exposures to overcome the lethality (see Materials and Methods). **A**, Rescue of LTD in *hsp70-akt;akt¹* after brief heat shock exposures. *hsp70-akt;akt¹* larvae were shifted to 18°C for 24–48 h after the last daily heat shock treatment (*hsp70-akt;akt¹*, 18°C). These larvae showed impaired LTD like the *akt* mutants ($p > 0.05$ compared with *akt⁴²²⁶* and *akt⁴²²⁶/akt¹*; two-way ANOVA). For acutely inducing *hsp70-akt* expression, a group of larvae was brought from 18°C to RT for 3–6 h and then were given heat shocks (18°C → HS; two heat shock exposures of 15 min at 37°C, 2 h interval). $n = 14$ and 9 for the groups of 18°C and 18°C → HS, respectively. **B**, Rescue of LTD by acutely induced *hsp70-akt* expression in the heterozygous allele *akt⁴²²⁶/akt¹*. The allele *hsp70-akt;akt⁴²²⁶/akt¹* at RT showed impaired LTD like the *akt* mutant (*hsp70-akt;akt⁴²²⁶/akt¹*; RT). The defective LTD was rescued by three to four brief heat shock exposures (37°C, 15 min, 1.5–2 h intervals) (*hsp70-akt;akt⁴²²⁶/akt¹*, RT → 3–4 HS). $n = 4$ and 6 for the groups RT and RT-HS, respectively. Calibration: 20 nA, 2 min. **C**, **D**, Rescue of LTD appears to correlate with the amount of *hsp70-akt* expression. **C**, In *hsp70-akt;akt¹* larvae, LTD was partially rescued at RT and fully rescued after additional heat shock exposures (2 times for 15 min, 37°C, 2 h interval). $n = 4$ and 5 for the RT and RT-HS groups, respectively. **D**, In *hsp70-akt;akt⁴²²⁶/akt¹*, impaired LTD was partially rescued after two heat shock exposures (15 min, 37°C, 2 h interval; $n = 3$) but was fully rescued after four heat shock exposures (see Fig. 6B; $n = 3$). $n = 4$ for the RT group.

candidate mechanism for increased mEJC frequency is an increase of the number of vesicles in RRP resulting from mobilization of alternative vesicle pools (e.g., RP) (Stevens and Sullivan, 1998; Waters and Smith, 2000; Tyler and Pozzo-Miller, 2001). It was shown that, at relatively high external Ca^{2+} concentration (2 mM), prolonged high-frequency stimulation (30 Hz, 30 s or more) mobilizes the RP via a cAMP/protein kinase A-dependent mechanism (Kuromi and Kidokoro, 2000; Kidokoro et al., 2004). Therefore, mobilization of synaptic vesicles from RP to RRP by the LTD-inducing tetanus may account for the increased mEJC frequency and contribute to evoked synaptic transmission after LTD induction. However, the Ca^{2+} concentration for LTD induction was much lower and the tetanic stimulation for inducing

LTD was shorter than required to mobilize the RP (Kuromi and Kidokoro, 2000). In addition, disrupting the cAMP signaling in *rutabaga* and *dunce* or by an inhibitor of protein kinase A (RP-cAMP; data not shown) did not significantly affect the increased mEJC frequency and LTD. Thus, mobilization of RP may not have occurred after LTD induction.

Akt and LTD

Akt mediates signaling from numerous growth factors, cytokines, hormones, and neurotransmitters to regulate diverse physiological functions, such as glucose metabolism, cell and organ growth, anti-apoptosis, and cell survival (Brazil and Hemmings, 2001). It also critically regulates neuronal survival (Dudek et al., 1997; Brunet et al., 2001) and the number of neurotransmitter (GABA) receptors (Wang et al., 2003). However, whether Akt mediates long-term synaptic plasticity has not been shown previously. Here we provided evidence that Akt is directly required to mediate LTD but not short-term synaptic plasticity. LTD was disrupted in multiple *akt* mutant alleles, *akt⁴²²⁶*, *akt⁴²²⁶/akt¹* (Fig. 5), and *hsp70-akt;akt¹* at 18°C (Fig. 7A), and was rescued by acutely induced expression of *hsp70-akt*. However, because no *akt* null allele is available, we are yet unable to address whether LTD would be abolished by complete loss of the Akt protein. It also remains to be determined whether *Drosophila* LTD is mediated by the same upstream (PI-3 kinase) and downstream (TOR) signaling of Akt as the metabotropic glutamate receptor-dependent LTD in the hippocampus (Hou and Klann, 2004). A few other Akt substrates [Raf, mitogen-activated protein kinase, nitric oxide synthase, and CREB (Brazil and Hemmings, 2001)] were also shown to be involved in LTD induction or expression in vertebrate synapses (Ito, 2001; Thiels et al., 2002); whether these molecules play a role in *Drosophila* LTD remains to be investigated.

In summary, we have described for the first time long-term synaptic depression at the *Drosophila* larval NMJ induced by specific high-frequency stimulation, which is directly mediated by Akt. Thus, it is possible to perform genetic analysis of the molecular mechanisms of long-term synaptic plasticity in *Drosophila*. Given the importance of long-term synaptic plasticity to learning and memory, our findings also suggest a role of Akt in these essential brain functions. Genetic analysis of long-term plasticity in *Drosophila* would reveal specific molecular events and interactions underlying behavioral plasticity.

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Figure 8. Normal synaptic transmission and short-term plasticity (*E–G*) in the *akt* mutants *akt⁴²²⁶* and *akt⁴²²⁶/akt¹*. *A*, Representative traces of spontaneous mEJCs in wild type (WT) and the *akt* mutants. Calibration: 1 nA, 1 s. *B*, Summary of amplitude and frequency of mEJCs. *n* = 8 and 6 for WT and *akt* groups, respectively. *C*, Representative traces of EJCs at different Ca^{2+} concentrations (0.1, 0.2, and 0.4 mM) in wild type and the *akt* mutants. Calibration: for 0.1 and 0.2 mM Ca^{2+} , 10 nA, 10 ms; for 0.4 mM Ca^{2+} , 20 nA, 10 ms. *D*, Ca^{2+} dependency of EJCs: logarithmic plot of the power relationship in the range of 0.1–0.4 mM Ca^{2+} . *n* = 6, 6, and 4 for the control (diamond), *akt⁴²²⁶* (square), and *akt⁴²²⁶/akt¹* (triangle) groups, respectively. *E*, Normal STF during a short train of repetitive stimulation (25 Hz) in the *akt* mutants. Top, Representative traces. Bottom, Summary of normalized EJC amplitude. *n* = 5 for each group. *F*, Normal dependence of STF on stimulation frequency in the *akt* mutants. Trains of 20 stimuli were delivered at the frequency of 0.5–20 Hz. The amplitudes of the last 10 responses (EJCs) in each train were averaged and normalized to the average EJC amplitude at 0.5 Hz. Top, EJC traces representative of the average of last 10 EJCs for 0.5 and 10 Hz. Calibration: 2 nA, 10 ms. Bottom, Summary. *n* = 5 for each group. *G*, Normal posttetanic potentiation in the *akt* mutants. Continuous recordings were made at 0.2 Hz stimulation before and after the 10 Hz tetanus. Top, Representative traces. Calibration: 5 nA, 1 min. Bottom, Summary of normalized EJC amplitudes. *n* = 11, 10, and 6 for control, *akt⁴²²⁶*, and *akt⁴²²⁶/akt¹* groups, respectively. [Ca^{2+}], 0.15 mM for *E–G*.

Figure 9. Expression of Akt at the *Drosophila* NMJ and reduced Akt expression in the hypomorphic *akt*⁴²²⁶ mutant. NMJs were stained using a polyclonal anti-dAkt antibody and FITC-conjugated secondary antibody. Shown in the top are representative fluorescence images of Akt immunostaining at the NMJ of wild type (WT) and *akt*⁴²²⁶. Under the color images are inverted images of the corresponding fluorescence images (see Materials and Methods). Comparison of staining intensity between wild type and *akt*⁴²²⁶ is shown in enlarged images of synaptic boutons and nerve branches (corresponding to the boxes in the fluorescence images and inverted images). Note that the difference in staining intensity between wild type and the mutant appears to be more apparent at the nerve branches. $n = 4$ and 3 for WT and *akt*⁴²²⁶, respectively. Scale bars, $20\ \mu\text{m}$.

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