Development/Plasticity/Repair

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 Ca2+ 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 dnce 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; Rohrbaugh 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; Rohrbaugh et al., 1999, 2000), such as dnce and rutabaga that express mutated forms of cAMP-specific phosphodiesterase and adenyl 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 dnce 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.
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 Ca2+-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 MgCl2, 10 NaHCO3, 5 trehalose, 5 HEPES, and 115 sucrose. For recordings, HL-3 solution was supplemented with CaCl2 (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, tetrodotoxin (TTX) of defined frequency and duration (see figure legends) was delivered after ~5 min of baseline stimulation. Methods for induction of short-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 Picosprizter 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 the Mini Analysis Program (Synaptosoft, Decatur, GA). 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
EJCss 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 [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 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...
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) decreased 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 (Rohrbourough et al., 1999), notch (Ge et al., 2004; Presente et al., 2004; Costa et al., 2005), gapI [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 akt<sup>4226</sup> and akt<sup>4226</sup>akt<sup>T</sup> (Fig. 5A). The hypomorphic allele akt<sup>4226</sup> harbors a P-element insertion upstream of the akt gene and was reported to cause reduced expression of akt (Spradling et al., 1999; Gao et al., 2000; Stocker et al., 2002), whereas akt<sup>T</sup> is an ethylmethyl sulfonate-induced null allele (embryonic lethal) because of a point mutation, dAktF327I, that confers a catalytically inactive kinase B/Akt.

We next asked whether the impaired LTD in the akt mutant is attributable to altered dependency on Ca<sup>2+</sup> concentration and tetanus frequency. Because LTD was most pronounced at 0.2 mM Ca<sup>2+</sup>, we examined LTD in akt<sup>4226</sup> 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. 6A, 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. 6C, 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 akt^{4226}/akt^{1} as in akt^{4226}. However, akt^{1} is a null allele whereas akt^{4226} is hypomorphic, so the trans-heterozygous allele should have less residual akt expression or activity than akt^{4226}. We therefore examined LTD in the heterozygous akt^{1/+} to test the dose requirement of Akt for LTD. LTD in akt^{1/+} 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 akt^{4226} 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 akt^{1/+}. These observations suggest that akt^{4226} is a strong akt mutant allele. Thus, akt^{4226} and akt^{4226}/akt^{1} 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 akt^{4226} 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 akt^{1} mutant carrying hsp70-akt (hsp70-akt;akt^{1}) 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;akt^{1}.

To examine hsp70-akt;akt^{1} 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;akt^{1} 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. Second, the hsp70 promoter may be leaky at room temperature. Third, we observed a few first-instar larvae homozygous for akt^{1} in hsp70-akt;akt^{1} 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 akt^{1} in hsp70-akt;akt^{1} (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
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 akt14226 (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 akt14226/akt1′ can also be rescued by transiently induced expression of hsp70-akt. We crossed the hsp70-akt;akt1′ with akt14226; 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; akt14226/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 EJC (Fig. 8C,D) in akt14226 and akt14226/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 akt14226, as shown in both the synaptic boutons and the nerve branches (Fig. 9). The staining intensity measured in the synaptic boutons of akt14226 was also significantly reduced compared with the wild type (wild type, 114.8 ± 3.1; akt14226, 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\textsuperscript{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 (e.g., 1 Hz for 15 min) was typically used to induce LTD, albeit at high external Ca\textsuperscript{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\textsuperscript{2+} concentrations. Although we used relatively high frequency stimulation (30 Hz), the relatively low external Ca\textsuperscript{2+} concentrations would limit Ca\textsuperscript{2+} influx; thus, similar internal Ca\textsuperscript{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 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 (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 ms Ca\textsuperscript{2+} when LTD was nearly absent (Fig. 1E). This short-term depression was also observed 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\textsuperscript{2+} concentration (e.g., 1.0 ms), 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
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 (Brazile 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 metabolotropic 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; Thiel 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.
References


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 Ca2+ concentrations (0.1, 0.2, and 0.4 mM) in wild type and the akt mutants. Calibration: for 0.1 and 0.2 mM Ca2+, 10 nA, 10 ms; for 0.4 mM Ca2+, 20 nA, 10 ms. D, Ca2+-dependency of EJCs: logarithmic plot of the power relationship in the range of 0.1–0.4 mM Ca2+. 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. ([Ca2+]i)0.15 mM for E–G.

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Figure 9. Expression of Akt at the Drosophila NMJ and reduced Akt expression in the hypomorphic akt mutant akt4226. NMJs were stained using a polyclonal anti-Akt 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 akt4226. Under the color images are inverted images of the corresponding fluorescence images (see Materials and Methods). Comparison of staining intensity between wild type and akt4226 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 akt4226, respectively.
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