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Pre- and post-synaptic mechanisms of synaptic strength homeostasis revealed by Slowpoke and Shaker K⁺ channel mutations in *Drosophila*

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Abstract

We report naturally occurring, systematic variations in synaptic strength at neuromuscular junctions along the dorsal-ventral (D-V) axis of the *Drosophila* larval body wall. These gradual changes were correlated with differences in presynaptic neurotransmitter release regulated by nerve terminal excitability and in postsynaptic receptor composition influencing miniature excitatory junctional potential (mEJP) amplitude. Surprisingly, synaptic strength and D-V differentials at physiological Ca²⁺ levels were not significantly altered in *slowpoke* (*slo*) and *Shaker* (*Sh*) mutants, despite their defects in two major repolarizing forces, Ca²⁺-activated Slo (BK) and voltage-activated Sh currents, respectively. However, lowering [Ca²⁺]_o levels revealed greatly altered synaptic mechanisms in these mutants, indicated by drastically enhanced EJPs in *Sh* but paradoxically reduced EJPs in *slo*. Removal of Sh current in *slo* mutants by 4-aminopyridine blockade or by combining *slo* with *Sh* mutations led to strikingly increased synaptic transmission, suggesting upregulation of presynaptic Sh current to limit excessive neurotransmitter release in the absence of Slo current. In addition, *slo* mutants displayed altered immunoreactivity intensity ratio between DGluRIIA and DGluRIIB receptor subunits. This modified receptor composition caused smaller mEJP amplitudes, further preventing excessive transmission in the absence of Slo current. Such compensatory regulation was prevented by *rutabaga* (*rut*) adenylyl cyclase mutations in *rut slo* double mutants, demonstrating a novel role of *rut* in homeostatic plasticity, in addition to its well-established function in learning behavior.

Keywords

Synaptic strength; dorsal-ventral differentials; *rutabaga* adenylyl cyclase; cAMP; Membrane excitability; Postsynaptic receptor composition

INTRODUCTION

Natural variations in synaptic strength, or differential responses of postsynaptic targets to presynaptic inputs, play crucial roles in matching the efficacy of individual synapses with different levels of activity demands. This mechanism is important for precise information

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transfer in neural circuits, in particular to support the functional organization of effectors in the various body plans. A well-studied example is the tonotopic processing of auditory information. There is a gradient of Ca^{2+} -activated BK or Slowpoke (Slo) K^+ channel-mediated I_{KCa} in sensory hair cells controlling tuning frequency along the base-to-apex axis of the cochlea (Fettiplace and Fuchs, 1999). In addition, differential expression of Shaker (Sh) voltage-activated K^+ (K_v1) channels contributes to tonotopic processing of auditory information corresponding to a gradient of membrane excitability and synaptic transmission in the chicken nucleus magnocellularis (Fukui and Ohmori, 2004) and in the rat lateral superior olive (Barnes-Davies et al., 2004). These examples highlight the importance of K^+ channels in establishing natural gradients of neuronal activity and synaptic strength.

Another important aspect of regulation of synaptic strength is homeostatic maintenance of synaptic strength in individual neurons within a particular circuit. Neurons are able to restore proper synaptic functions when the neural circuits are destabilized following perturbations in membrane excitability, in neurotransmitter release machinery, in postsynaptic receptor functions, or in synaptic growth. For example, chronic blockade by TTX or enhancement by GABA receptor antagonists of mammalian neuronal activity in culture leads to a compensatory increase or decrease in postsynaptic response amplitude via regulation of AMPA receptor accumulation (O'Brien et al., 1998, Turrigiano et al., 1998). Similar homeostatic regulation occurs at *Drosophila* neuromuscular junctions. A change in postsynaptic activity by expressing a persistent outward K^+ current, causing a hyperpolarized resting potential, results in a compensatory increase in presynaptic neurotransmitter release (Paradis et al., 2001). Mutations that decrease postsynaptic receptor density lead to an increase in presynaptic neurotransmitter release (Petersen et al., 1997). In addition, compensatory increases in synaptic areas and active zone numbers are induced by reduced number of synaptic varicosities to match the demand of target muscles (Stewart et al., 1996).

It has not been established how differential synaptic strength according to different body plans is related to homeostatic mechanisms following perturbations of synaptic strength. Here we document naturally occurring variations in synaptic strength along the dorsal-ventral (D-V) body axis in the body wall of *Drosophila* larvae that correlate with differential expression of Sh K^+ currents affecting presynaptic neurotransmitter release and with regulation of postsynaptic receptor composition. Mutational and pharmacological analyses reveal that, in the absence of major repolarizing Slo K^+ channels, synaptic strength and its D-V differentials are still maintained homeostatically through presynaptic upregulation of Sh currents and postsynaptic alterations in receptor composition. Mutations in *rutabaga* (*rut*) adenylate cyclase (AC) prevent these homeostatic adjustments in *rut slo* double mutants, indicating that cAMP is central to the homeostatic plasticity in addition to its well-known roles in behavioral plasticity.

EXPERIMENTAL PROCEDURES

Fly stocks

The fly stocks used in this study include: wild type (WT) Canton-S (CS); K^+ channel mutants, including *slowpoke* (*slo*) (*slo¹*, *slo⁹⁸*, and *slo⁴*), *Shaker* (*Sh*) (*Sh^M* and *Sh¹³³*), and *Sh;;slo* (*Sh¹³³;;slo¹* and *Sh^M;;slo⁹⁸*), and AC mutants *rut* (*rut¹* and *rut¹⁰⁸⁴*) and *rut slo* (*rut¹;;slo¹* and *rut¹;;slo¹/slo⁴*). Data collected from different alleles of *slo* and *Sh* indicate similar physiological phenotypes (see Table S1 in the Supplement Data). Thus, results from the different alleles are combined in analysis to increase statistical power. All these stocks were raised in the presence of conventional fly medium and maintained at room temperature.

Preparations and Electrophysiology

Preparation of wandering third instar larvae and intracellular recordings of excitatory junctional potentials (EJPs) were performed as described previously (Ueda and Wu, 2006). Evoked EJPs upon segmental nerve stimulation were recorded from abdominal segments 3 through 6, mostly from 4th and 5th segments, in HL3.1 saline (Feng et al., 2004) containing 0.2 mM of Ca²⁺, unless specified. For the analysis of D-V disparity, EJPs were sampled from multiple muscles in a single hemisegment (mostly from the 4th and 5th abdominal segments) per larva (Fig. 1B) to ensure stable muscle conditions throughout the experiment. The EJP amplitude from muscles 6/7 (V) was divided by that from muscle 1/9 (D) within the same hemisegment of body wall to calculate the EJP ratio (V/D). Miniature EJPs (mEJPs) with the resting membrane potential lower than -60 mV were analyzed by MiniAnalysis software (Synaptosoft Inc., Fort Lee, NJ, USA). Quantal content was calculated either by dividing the mean EJP amplitude by the mean mEJP amplitude at 0.2 mM Ca²⁺, or by calculating the log (1/failure rate) during 200 consecutive stimuli in the presence of 0.1 mM Ca²⁺. The correction procedure for nonlinear summation of synaptic potential (Martin, 1955) was applied for EJP ratio (V/D), quantal content, and drug-induced EJP change. All EJP data were collected using CLAMPEX (version 5.5, Molecular Devices Corporation, Sunnyvale, CA, USA) and analyzed using CLAMPFIT (version 6.0, Molecular Devices Corporation) and Origin software (version 6.0, OriginLab Corporation, Northampton, MA, USA). For selective blockade of Slo and Sh K⁺ channel function, ChTx (50 nM, Alomone Labs, Ltd., Jerusalem, Israel) and 4-AP (0.2 mM, otherwise indicated, Sigma-Aldrich Corp., St. Louis, MO, USA), respectively, were applied in the bath saline at least for 2 min before data collection.

Immunohistochemistry

Dissected larvae were fixed with either 100% ice-cold methanol for 5 min for immunostaining of DGluRIIA, otherwise with 3.7% formaldehyde in phosphate buffer saline for 20 min. The primary antibodies used include: monoclonal FasII (1D4, 1:4, Developmental Studies Hybridoma Bank (DSHB), Univ. of Iowa) and DGluRIIA antibody (8B4D2, 1:10, DSHB, Univ. of Iowa), and rabbit polyclonal Dlg (1:20,000, Dr. V. Budnik at Univ. of Massachusetts, Worcester), DGluRIIB (1:1000) and DGluRIIC (1:2000) antibody (Dr. A. DiAntonio at Washington Univ., St. Louis). The fluorescent secondary antibodies at 1:50 or 1:200 were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). For direct comparison of DGluRIIA, DGluRIIB, and DGluRIIC density among different genotypes, all larvae were treated within single tubes and washout chambers throughout the entire procedures.

Imaging and Analysis

Images of NMJs on muscles 6/7 (ventral) and 1(dorsal) from abdominal segments 3 through 6 were collected using a M1024 confocal microscope (BioRad, Hercules, CA, USA) with an oil-immersed 60X objective. The average pixel intensity of fluorescent signals was measured from the collapsed Z-stacked images using Image J software (NIH image, USA) by outlining the boundary of the entire type Ib boutons (Atwood et al., 1993). The boundary was determined by enclosing the area of pixel intensity at least 30% beyond the background level. The D/V ratio of pixel intensity was then calculated from the pixel intensity measured from dorsal (M1) and ventral (M6/7) NMJs within the same hemisegment.

For direct comparison across genotypes of DGluRII subunit density, settings for exposure and reading were fixed for all larvae in the same batch processed and stained simultaneously. The intensity readings were normalized to the average value from the ventral NMJs of WT (see Figs. 4 and S1 in the Supplement Data). The DGluRIIA/

DGluRIIB (IIA/IIB) ratio was compared between double-labeled ventral and dorsal NMJs and across genotypes.

Statistical Analyses

For comparison between ventral and dorsal NMJs within a genotype, or between two genotypes, two-tailed *t*-test was routinely carried out against the null hypothesis of equal values between NMJs or genotypes. When each value was compared among more than two genotypes, one-way ANOVA was carried out with the tests for equal variance and multiple comparisons. In both cases, *P*-value less than 0.05 was considered to be significantly different. All statistical analyses were performed using Origin software (version 6.0, OriginLab Corporation).

RESULTS

Naturally occurring, systematic variations in synaptic strength along the dorsal-ventral body axis and the potential pre- and post-synaptic mechanisms

Neuromuscular junctions (NMJs) formed in the body-wall musculature of the third instar larvae of *Drosophila* have been one of the best-studied systems for synaptic transmission. The arrangement of body-wall muscles in each abdominal hemisegment is well characterized, consisting of 30 muscles in each segment (Fig. 1A). Each muscle is poly-innervated by multiple axons that form synaptic boutons along terminal branches, which differ in their shape and size (Johansen et al., 1989). Among these muscles, ventral longitudinal muscles including muscles 7, 6, 13, and 12 have been most frequently utilized for the effects of mutations on synaptic responses or excitatory junctional potentials (EJPs). In contrast, synaptic transmission in other muscles in the hemisegment, including the dorsal musculature, has not been described in detail.

In this study, we found a systematic variation in evoked synaptic responses among NMJs of the identified muscles within a single hemisegment of WT larvae. Synaptic strength indicated by EJP amplitude at 0.2 mM $[Ca^{2+}]_o$ in HL3.1 saline was consistently greater in ventral muscles, represented by muscle 6, than in dorsal muscles, represented by muscle 1 (Fig. 1A). The pooled data from abdominal longitudinal muscles (Fig. 1B, 6 to 13 larvae) show a trend of increasing EJP amplitudes from dorsal to ventral midlines. Notably, the diagonal muscles closest to the ventral midline, muscles 15 and 16, actually displayed EJPs of smaller amplitudes.

To explore the potential mechanisms of this profile of synaptic variation, we carried out quantal analysis that reveals differences in quantal size (mEJP amplitude or the postsynaptic response to a single vesicle release) and quantal content (the number of presynaptic vesicle released). Significantly, mEJP amplitude was larger in ventral (muscle 6 or 7, or M6/7) than dorsal (muscle 1 or 9, or M1/9) muscles (see Fig. 1C for two sample traces and 1D for the pooled data, $P < 0.05$), parallel to the observed EJP amplitude (Fig. 1B). Quantal content was subsequently determined for ventral and dorsal NMJs. EJP amplitudes were corrected for non-linear summation to estimate the quantal content accurately (see Experimental procedures). Our results demonstrate that the EJP quantal content was higher at ventral than dorsal NMJs (Fig. 1D, M6/7 vs. 1/9, $P < 0.01$).

Recent studies have proposed a link between quantal size and glutamate receptor subunit composition at *Drosophila* larval NMJs. The relative abundance of DGluRIIA and DGluRIIB, two of the five identified glutamate receptor subunits in *Drosophila* (Schuster et al., 1991, Petersen et al., 1997, Featherstone et al., 2005, Qin et al., 2005), influences the quantal size (Petersen et al., 1997, DiAntonio et al., 1999, Marrus et al., 2004). Indeed, our results support a close relationship between receptor composition and quantal size. A clear

dorsal-ventral (D-V) difference in receptor composition was observed when the pixel intensity ratio between DGluRIIA and DGluRIIB immunoreactivity (IIA/IIB) was calculated from the more prominent type Ib boutons in multiple NMJs within a single hemisegment (see Experimental procedures) (Fig. 1E). The ratios in type Is boutons co-varied with those in type Ib boutons at the same NMJs (data not shown). The IIA/IIB ratio difference along the D-V axis was mostly due to the varying density of DGluRIIA without significant variations in DGluRIIB (see Fig. S1 in the Supplement Data). It is noteworthy that a greater ratio was obtained from M6/7 than M1/9, consistent with their quantal size difference. Overall, the pooled data from 17 hemisegments in 5 simultaneously processed larvae displayed a gradual change in the IIA/IIB ratio along the D-V axis despite apparent deviations in muscles 15/16 and 13 (Fig. 1E). This profile of D-V receptor composition paralleled that of EJP amplitude (Fig. 1B).

Therefore, our analysis for quantal release and immunostaining suggests distinct pre- and post-synaptic mechanisms that are coupled with the D-V differences in synaptic strength, i.e. regulation of presynaptic neurotransmitter release, or quantal content, and postsynaptic receptor composition influencing mEJP amplitude, or quantal size.

D-V differences in synaptic protein expression

Further immunohistochemical analysis indicated that the D-V difference in the receptor density is restricted to DGluRIIA (Schuster et al., 1991) and, to a lesser extent, DGluRIIB (Petersen et al., 1997), but not DGluRIIC, one of the shared subunits among glutamate receptor subtypes (Featherstone et al., 2005, Qin et al., 2005). The D-V difference in receptor density was represented by relative pixel intensity of M1 over M6/7 for each subunit within the same hemisegment (see Experimental procedures). Our results indicate a D/V ratio consistently smaller than 1.0 for DGluRIIA (Figs. 2A and 2E), but slightly higher than 1.0 for DGluRIIB subunits (Figs. 2B and 2E). In contrast, DGluRIIC density did not differ between ventral and dorsal NMJs (Fig. 2E), suggesting similar density of total glutamate receptor subtypes at both NMJs.

Comparisons of D/V density ratios for other synaptic proteins also revealed a differential distribution of an adhesion molecule, Fasciclin II (FasII) (Bastiani et al., 1987) (Fig. 2C), of which expression is affected by varying activity levels at *Drosophila* larval NMJs upon environmental or genetic manipulations (Schuster et al., 1996b, Sigrist et al., 2003). The D/V ratio was consistently greater than 1.0 for FasII in all preparations examined, indicating higher density at dorsal NMJs (Fig. 2E, $P < 0.001$). In contrast, Discs-Large (Dlg) (Lahey et al., 1994, Budnik et al., 1996), an invertebrate scaffold protein homologous to PSD-95 required for clustering of FasII and Sh K⁺ channels (Tejedor et al., 1997, Thomas et al., 1997, Zito et al., 1997), did not show any D-V density differences (Figs. 2D and 2E). Furthermore, the immunoreactivity to anti-HRP, which recognizes pan-neuronal markers in insects (Jan and Jan, 1982), showed a slightly higher intensity at dorsal NMJs (Fig. 2E, $P < 0.01$). However, HRP density did not co-vary with FasII density in double-labeled preparations (data not shown).

Such a D-V difference in FasII density led us to examine the possibility that it contributes to the functional synaptic variation (Fig. 1). However, *fasII* mutants displayed D-V differences in both EJP amplitudes and receptor density similar to WT (data not shown). Therefore, differential distribution of FasII is not required for the D-V disparity in synaptic strength described above, supporting the idea that FasII is involved in plasticity of NMJ morphology, but not synaptic efficacy (Schuster et al., 1996a, Schuster et al., 1996b).

Effects of elimination of Ca^{2+} -activated Slo and voltage-activated Sh K^+ channels on synaptic strength

We then examined how the pre- and post-synaptic mechanisms described above are affected by changes in activity level using K^+ channel mutations. Among K^+ channels, the Ca^{2+} -activated Slo and voltage-activated Sh K^+ channels have been first characterized molecularly in *Drosophila* (Kamb et al., 1987, Papazian et al., 1987, Atkinson et al., 1991) and their mutational effects on larval neuromuscular transmission have been well described (Jan et al., 1977, Wu et al., 1983, Gho and Ganetzky, 1992, Warbington et al., 1996). Sh I_A is important for axonal repolarization and nerve terminal excitability control (Tanouye et al., 1981, Ganetzky and Wu, 1983, Tanouye and Ferrus, 1985, Ueda and Wu, 2006). Slo (BK) channels are co-localized at presynaptic terminals with voltage-gated Ca^{2+} channels, a major source of Ca^{2+} influx for transmitter release (Robitaille et al., 1993, Yazejian et al., 1997, Marrion and Tavalin, 1998), and thus provide an effective feedback mechanism to limit Ca^{2+} influx-induced transmitter release. We first examined how these two important repolarizing channels affect natural variations in synaptic strength along the D-V axis.

Surprisingly, at a physiological $[\text{Ca}^{2+}]_o$ level (0.5 mM or higher), EJP amplitude and D-V differences in *slo* and *Sh* mutants were not severely affected as compared to WT (Fig. 3A, top traces; see Fig. S2 in the Supplement Data for the pooled data), contrary to the expected effects from a loss of major repolarizing force to stabilize membrane excitability. It should be noted that under this condition (0.5 mM Ca^{2+} in HL3.1 saline) EJP amplitudes are closer to the reported response amplitudes obtained from HL3 saline containing 1.5 mM of Ca^{2+} (Stewart et al., 1994, Feng et al., 2004). EJPs described here did not reach saturation, as indicated by further increase in the amplitude at even higher Ca^{2+} levels (1.0 or 1.5 mM), and displayed considerable variations in the amplitudes (Fig. S2 in the Supplement Data). Therefore, these results indicate that these *slo* and *Sh* mutant NMJs can indeed perform normal synaptic function under a physiological condition, suggesting the presence of homeostatic mechanisms to compensate the loss of these important K^+ channel functions.

In contrast to a lack of clear mutant phenotypes at physiological Ca^{2+} levels, striking mutant phenotypes were revealed at low $[\text{Ca}^{2+}]_o$ (0.2 mM) in *Sh* and *slo* larvae (Fig. 3A, bottom two traces). Consistent with previous reports (Jan et al., 1977, Wu et al., 1983, Ueda and Wu, 2006), *Sh* mutations induced a significant increase in EJP amplitude at both ventral and dorsal NMJs, as expected from enhanced nerve excitability (Fig. 3A). At this Ca^{2+} level, *Sh* EJPs were more than 4-fold greater than WT EJPs, or greater than 10-fold in transmitter release when non-linear correction of EJPs was considered (Fig. 3B). However, in *slo* mutants, we found a significant reduction, but not enhancement, in EJPs at ventral NMJs (Figs. 3A and 3B). This paradoxical result, also described in a previous report (Warbington et al., 1996), is contrary to the expected increase in membrane excitability due to a loss of Slo channels. In addition to altered EJP amplitudes, comparing the D-V muscle pairs (M6/7 for ventral (V) and M1/9 for dorsal (D) NMJs) for individual larvae revealed disruptions in the D-V difference in synaptic strength in *Sh* and *slo* mutants (EJP amplitude in Fig. 3B and the V/D ratio in Fig. 3C). Despite variable amplitudes among preparations, D-V pairs from the same hemisegment connected by lines nevertheless showed a clear difference in WT (Fig. 3B, $P < 0.001$, V vs. D at 0.2 mM $[\text{Ca}^{2+}]_o$). Fig. 3B also demonstrates a preferential effect of *Sh* mutations on dorsal EJPs in addition to the average increase in synaptic strength ($P < 0.0001$, WT vs. *Sh*). On average, EJP amplitude was increased to a greater extent at dorsal NMJs with some extreme cases of dorsal EJPs becoming greater than ventral EJPs (see Fig. 3A, bottom traces for an example; see also lines with positive slopes in Fig. 3B and columns left to the vertical line in Fig. 3C), resulting in no clear overall D-V difference in mean EJP amplitude. On the other hand, *slo* EJP amplitude was reduced, mostly at ventral NMJs (Fig. 3B, $P < 0.005$, WT vs. *slo* ventral NMJs). Again, in some cases of *slo* mutants, dorsal, but not ventral, NMJs displayed greater EJPs (bottom traces in Fig. 3A; see also Figs.

3B and 3C). These extreme cases of reversed D-V differentials were also found in mutants at different levels of low $[Ca^{2+}]_o$ (see Fig. 3C for *Sh*, 0.1 and 0.2 mM), but have not been observed in WT.

Modification of synaptic protein distribution and postsynaptic receptor composition in *slo* mutants

Disrupted D-V differentials at *slo* and *Sh* NMJs revealed at low $[Ca^{2+}]_o$ levels (Fig. 3) suggest that these two major repolarizing K^+ channels may play important roles in the D-V profile of synaptic strength. We first examined whether there are changes in distribution of synaptic proteins in the mutants as compared WT. Fig. 4D depicts mean of D/V ratios of synaptic protein density as well as SD to indicate the range of the variability. In *Sh* mutants, we did not detect significant changes in D/V ratios of the proteins from those shown in Fig. 2 for WT, except for Dlg (Figs. 4A–D).

In contrast, *slo* mutations induced clear changes in the distribution of FasII and Dlg compared to WT. We found in *slo* mutants a significant reduction in D-V differences for FasII and differential D-V intensities for Dlg that was evenly distributed in WT (Figs. 4A₁, 4C₁, and 4D). The significance of these alterations remains unclear. As mentioned above, synaptic strength as well as glutamate receptor density in *fasII* and *dlg* mutants were not different from WT (data not shown). Thus, it is unlikely that altered distributions of FasII and Dlg in *slo* mutants correlate with the profile of synaptic strength. Whether modified distributions of these molecules in *slo* mutants can lead to alterations in synaptic features other than transmission efficacy needs to be further investigated in future studies.

Significantly, *slo* mutations induced a change in DGluRIIA density along the D-V axis. While there was no change in the D/V ratio of DGluRIIB and DGluRIIC densities (Fig. 4D), it was significantly reduced for DGluRIIA density in *slo* mutants (Figs. 4B₁ and 4D, $P < 0.05$ for WT vs. *slo*). Calculation of DGluRIIA/IIB (IIA/IIB) ratio at ventral (M6/7) and dorsal (M1) NMJs within the same hemisegment again indicated a preferential effect of *slo* mutations on ventral NMJs, where the IIA/IIB ratio was more drastically decreased than at dorsal NMJs compared to WT and *Sh* (Fig. 4E, see also Figs. S1 and S3 in the Supplement Data for the relative density of DGluRIIA and DGluRIIB at double-stained NMJs and IIA/IIB ratio throughout a single hemisegment, respectively).

Modification of quantal size and quantal content by *slo* and *Sh* mutations

The preferential effect of *slo* mutations on receptor composition (IIA/IIB ratio) at ventral NMJs was reflected by a clear reduction in quantal size at ventral, but not dorsal, NMJs (Fig. 5, $P < 0.01$ for WT vs. *slo*, $P < 0.05$ for *Sh* vs. *slo*). While mEJP amplitudes were larger at ventral than dorsal NMJs in both WT and *Sh* larvae ($P < 0.05$), such difference disappeared in *slo* mutants (Figs. 5A and 5B, top panel). Together with a more drastically reduced IIA/IIB ratio at ventral NMJs (Fig. 4E), these results demonstrate a postsynaptic modification that leads to smaller EJP amplitudes in *slo* mutants at lower Ca^{2+} levels (Fig. 3).

One important feature of *Sh* mutations is the enhanced EJPs, more extreme at dorsal NMJs, at 0.2 mM $[Ca^{2+}]_o$ (Fig. 3). Significantly, unaltered receptor composition and quantal size in *Sh* mutants (Figs. 4E, 5A, and 5B, top panel) gave rise to extremely large quantal content when quantal analysis was performed (see Experimental procedures). Notably, the resultant quantal contents for ventral and dorsal EJPs were equally large, whereas in WT dorsal quantal content was about 3-fold smaller (Fig. 5B, bottom panel, V vs. D, $P < 0.01$). Unexpectedly, quantal content in *slo* mutants was not significantly different from WT in the absolute scale (Fig. 5B, bottom panel). Similar quantal contents between WT and *slo* were also observed upon failure analysis (data not shown, see Experimental procedures). This

unexpected result, plus *Sh*-induced increases in quantal content and EJP amplitude at dorsal NMJs, led us to examine a potential presynaptic mechanism for the homeostatic regulation of synaptic strength in *slo* and for the natural D-V disparity in synaptic strength, i.e. differential Sh current expression (Fig. 3A, see below).

An important clue to a higher abundance of presynaptic Sh currents at dorsal NMJs was discovered when WT preparations were treated with 4-aminopyridine (4-AP), a selective blocker for Sh I_A . Upon removal of Sh I_A , EJPs at dorsal NMJs increased proportionally greater than those at ventral NMJs (Fig. 6A, left). Furthermore, pooled data from seven larvae indicated a decrease in the EJP ratios (V/D) following 4-AP treatment (Fig. 6A, right). Therefore, the synaptic transmitter release capability of dorsal NMJs is at least comparable to that of ventral NMJs once Sh I_A is removed. The results collectively support the hypothesis that higher expression of Sh current at dorsal NMJs more effectively restricts transmitter release and hence causes lower quantal content. Together with smaller quantal size, suppressed quantal content by upregulated Sh I_A may thus underlie smaller EJPs at dorsal NMJs in WT.

Homeostatic upregulation of presynaptic Sh I_A in *slo* mutants

In vertebrate species, it is established that Slo (BK) channels, co-localized at presynaptic terminals with voltage-gated Ca^{2+} channels, act as a major transmitter release regulator (Robitaille et al., 1993, Yazejian et al., 1997, Marrion and Tavalin, 1998). It is therefore surprising that lack of BK currents in *slo* mutants did not cause greatly enhanced synaptic transmission (Fig. 3) with similar amount of transmitter release, as indicated by comparable quantal contents between WT and *slo* at a low Ca^{2+} level (0.2 mM, Fig. 5B, bottom). These observations raise a possibility of a compensatory mechanism that minimizes the effect of a loss of Slo channels to retain synaptic function at physiological Ca^{2+} levels.

We found evidence of upregulated Sh I_A in *slo* mutants when we examined changes in EJPs following 4-AP treatment. Serial application of 4-AP with increasing concentrations revealed a far greater effect in *slo* than WT. Enhancement of EJPs in WT was detectable at 0.02 mM of 4-AP, but reached a saturated level beyond 0.1 mM (Fig. 6B, Δ EJP). In contrast, EJPs in *slo* mutants continued to increase without a clear saturation effect up to 1.0 mM, and therefore the EJP increment became progressively greater than that in WT (Fig. 6B, at 0.2 mM or higher [4-AP], $P < 0.001$ or $P < 0.01$). Remarkably, at higher 4-AP concentrations, EJP amplitude of *slo* mutants became larger than that of WT (data not shown). It is important to note that 4-AP treatment in WT did not enhance EJP amplitude beyond the extent displayed by *Sh* mutants at different Ca^{2+} concentrations and that 4-AP treatment did not further increase EJPs in *Sh* mutants (data not shown). These imply that a major portion of I_A in presynaptic terminals is mediated by Sh rather than Shal (Covarrubias et al., 1991) and that the observed 4-AP effects reflect manipulation of mainly Sh I_A .

The functional significance of increased Sh I_A currents in *slo* mutants was demonstrated in *Sh;;slo* double mutants and 4-AP-treated *slo* single mutants. Upon elimination of Sh I_A in *slo* mutants, EJPs were significantly prolonged along with the occurrence of supernumerary EJPs following a single stimulus at both low and physiological $[Ca^{2+}]_o$ (Fig. 6C). These phenotypes, characteristic of presynaptic hyperexcitability (Ganetzky and Wu, 1983), were also reproduced by combination of Charybdotoxin (ChTx), a selective blocker of Slo current, with 4-AP in WT, or by ChTx treatment in *Sh* mutants (Fig. 6C), confirming both Slo and Sh as major K^+ currents controlling presynaptic excitability. It is therefore necessary to compensate the loss of BK currents in *slo* mutants by a major repolarization force, in this case, Sh I_A , which prevents excessive vesicle release.

cAMP-dependent homeostatic regulation of synaptic strength in *slo* mutants

Functions of ion channels, including *Slo* and *Sh*, are often regulated by second messenger pathways such as the cAMP-dependent cascade (Zhong and Wu, 1993, Esguerra et al., 1994, Jonas and Kaczmarek, 1996). On the other hand, activities of these pathways are regulated in Ca^{2+} - and activity-dependent manners, e.g. during induction of Ca^{2+} -dependent LTP, involving delicate control of Ca^{2+} influx by voltage- and Ca^{2+} -activated ion channels (Ferguson and Storm, 2004). We found that both pre- and post-synaptic homeostatic mechanisms activated in *slo* mutants (Figs. 4, 5, and 6) are affected by mutations of *rut* encoding a Ca^{2+} /CaM-dependent AC that produces cAMP in a Ca^{2+} -dependent manner (Dudai et al., 1983, Livingstone et al., 1984). We observed in *rut^l* mutants greatly reduced EJP amplitude (to a lesser extent in *rut¹⁰⁸⁴*, data not shown) at both ventral and dorsal NMJs compared to WT (Figs. 7A and 7B, $P < 0.01$), consistent with reduced Ca^{2+} influx in *rut* presynaptic terminals (Ueda and Wu, 2003). Nevertheless, the *rut* mutants displayed a clear D-V difference in EJP amplitude (Fig. 7B, V vs. D, $P < 0.01$).

We first examined the effect of *rut* on homeostatic mechanisms in *rut;;slo* double mutants at low $[\text{Ca}^{2+}]_o$, at which *slo* phenotypes were most striking (Fig. 3). Surprisingly, the average EJP amplitude of *rut;;slo* double mutants was greater than either single mutants (Fig. 7A) and with WT-like D-V differences (Fig. 7B, V vs. D, $P < 0.01$). The enhancement of EJP amplitude was more clearly observed at ventral NMJs (Fig. 7B, *slo* vs. *rut;;slo*, $P < 0.001$), where *slo* mutant phenotypes were more striking in the homeostatic modifications of quantal size and receptor composition, i.e. the IIA/IIB ratio (Figs. 4 and 5).

We found that *rut* mutants retained a similar IIA/IIB ratio and quantal size (Fig. 7C) comparable to WT. However, in *rut;;slo* double mutants, both the IIA/IIB ratio and quantal size were increased compared to *slo* (Fig. 7C, *slo* vs. *rut;;slo*, $P < 0.05$ for IIA/IIB ratio, $P < 0.001$ for quantal size). There was a clear correlation between these two parameters as we observed the same descending order of *rut*, *rut;;slo*, and *slo* in their values. The action of *rut* AC in this case is likely postsynaptic since IIA/IIB ratios can vary even among NMJs with a common presynaptic motoneuron identity (i.e. innervation of type Is branches in M6/7, 13, and 12), as mentioned above.

In addition to such postsynaptic modification, a presynaptic role for *rut* was also suggested by comparisons in quantal content and 4-AP response between *slo* single and *rut;;slo* double mutants (Figs. 7D and 7E). Quantal content was significantly lower in *rut^l* mutants (Fig. 7D, $P < 0.05$, *rut* vs. WT), as deduced from smaller EJP amplitude with unaltered quantal size (Figs. 7B and 7C, right panel). However, quantal content of *rut;;slo* double mutants appeared to be enhanced compared with the single mutants (Fig. 7D, $P < 0.05$ for *rut* vs. *rut;;slo*, $P < 0.07$ for *slo* vs. *rut;;slo*). Importantly, such enhancement may involve *Sh* I_A adjustment since application of 4-AP (0.2 mM) in *rut;;slo* mutants no longer yielded an extreme EJP increment as observed in *slo* single mutants (Fig. 7E, $P < 0.05$ for *slo* vs. other genotypes; see also Fig. 6B). Therefore, our results indicate that, together with postsynaptic modifications in receptor composition and quantal size, presynaptic upregulation of *Sh* current triggered by *slo* mutations may also involve cAMP-dependent pathways.

DISCUSSION

Synaptic strength profile along the D-V axis of larval body-wall musculature and the roles of *Slo* and *Sh* K^+ currents in its homeostatic maintenance

Systematic variations in synaptic strength according to the body plans provide mechanisms for ordered information transfer in neural circuits. In this study, we, for the first time, analyzed a novel D-V disparity in synaptic strength at *Drosophila* larval body-wall NMJs. In *Drosophila* larvae, a variation along the anterior-posterior (A-P) axis has been reported for

the number of synaptic boutons (Budnik et al., 1990). While the A-P variation in synaptic morphology is derived from motoneurons of the same identities among body segments, the D-V differential in synaptic strength involves motoneurons of different identities within the same segment (Johansen et al., 1989, Landgraf et al., 1997). It is worth pointing out that the D-V profile of EJP amplitude does not correlate with the number of boutons in different muscles (data not shown). In addition, we did not find receptor composition differences for identified NMJs along the A-P axis (see Fig. S4 in the Supplement data), in contrast to gradual changes along the D-V axis (Fig. 1E), highlighting two types of morphological and systematic functional variations. Equipped with microchetae on the ventral cuticle, ventral muscles likely face greater mechanical demands upon crawling than dorsal muscles, which may require different levels of synaptic strength.

Fig. 8A summarizes the findings and integrates the plausible pre- and post-synaptic mechanisms that may underlie the natural variations in synaptic strength. Analysis of the D-V profile of synaptic strength *in vivo* indicated a potential physiological significance of postsynaptic glutamate receptor composition (DGluRIIA/IIB) (Fig. 8A). With shared subunits, e.g. DGluRIIC, competition between DGluRIIA and DGluRIIB for receptor assembly is thought to determine single channel conductance and kinetics, and thus quantal size (Petersen et al., 1997, DiAntonio et al., 1999). Indeed, we found D-V difference in quantal size correlated with differential IIA/IIB ratios, but not DGluRIIC density, at both type Ib (Figs. 1, 2, and 5) and Is NMJs (data not shown), providing a strong support for the proposed functional consequences of subunit competition. In addition to such postsynaptic mechanism, regulation of presynaptic excitability by differential expression of Sh current also contributes to the D-V differentials in synaptic strength (Fig. 8A). This is supported by the stronger effect of 4-AP as well as *Sh* mutations at dorsal than ventral NMJs (Figs. 3, 5B, and 6A).

These pre- and post-synaptic mechanisms are homeostatically modified in *slo* mutants to retain synaptic function at physiological Ca^{2+} levels (Fig. 8B, 0.5 mM). Sh I_A acts as a major repolarizing force at low $[\text{Ca}^{2+}]_o$ (Fig. 5B), where repolarization provided by Ca^{2+} -activated Slo current is minimal. Enhanced neurotransmitter release in *Sh* mutants is most striking at low $[\text{Ca}^{2+}]_o$, but progressively less obvious as $[\text{Ca}^{2+}]_o$ rises to physiological levels (Fig. 3A), where Slo current becomes full-blown. This suggests a contrasting role for Slo current at higher $[\text{Ca}^{2+}]_o$ in controlling presynaptic excitability (Fig. 8B, left, dark blue), as indicated by striking presynaptic hyperexcitability induced by removal of Slo in *Sh* mutants (Fig. 6C). The fact that *slo* mutants do not display obvious abnormalities in EJPs at physiological $[\text{Ca}^{2+}]_o$ (Fig. 8B, right) indicates the existence of homeostatic restoration mechanisms. At lower $[\text{Ca}^{2+}]_o$ (0.2 mM, light blue), the paradoxical reduction in *slo* EJP amplitude is resolved by a presynaptic upregulation of compensatory repolarizing forces, Sh I_A (Fig. 8B, right), supported by greater effect of 4-AP on EJP amplitude in *slo* compared to WT (Fig. 6B). Additional modifications in postsynaptic receptor composition were also found in *slo*, contributing to reduced quantal size (Figs. 4E, 5 and 8B, right). The combined effects of decreased quantal size and nearly normal quantal content at lower $[\text{Ca}^{2+}]_o$ lead to a smaller EJPs, but result in almost normal EJP amplitude at physiological $[\text{Ca}^{2+}]_o$ (Fig. 8B, right).

Activation of cAMP-dependent homeostatic mechanisms

Our finding of upregulated Sh I_A in *slo* presynaptic terminals is an interesting parallel to the recent report in cultured *Drosophila* embryonic neurons that reduced Slo current following the loss of a major Ca^{2+} current is compensated by upregulated Sh I_A (Peng and Wu, 2007). These observations in *Drosophila* corroborate previous reports that neuronal excitability is homeostatically regulated through modulation of ionic currents in response to manipulations of a specific membrane conductance (Marder and Goaillard, 2006). For example,

overexpression of I_A in crustacean stomatogastric ganglion neurons induces concomitant increase in the hyperpolarization-activated current, with an end result of maintained neuronal firing patterns (MacLean et al., 2003). In mutant mice defective in $Ca_v2.1$ channels, total Ca^{2+} current density in forebrain neurons remains unaltered due to compensatory increase in Ca_v1 activity (Etheredge et al., 2007).

These homeostatic mechanisms may require long-term modifications of pre- or post-synaptic machinery. Acute application of ChTx to block Slo channels in WT at low $[Ca^{2+}]_o$ failed to reproduce the characteristic *slo* mutant phenotypes of reduced EJP amplitudes (data not shown). Similarly, compensatory upregulation of Sh current in cultured neurons also requires chronic reduction of Ca^{2+} currents (Peng and Wu, 2007). Thus, these observations suggest that chronic, but not acute, dysfunction of ion channels is required to induce homeostatic compensation.

Our results demonstrate the involvement of a prevalent signal transduction pathway, the cAMP cascade, in synaptic homeostasis. It is well known that the cAMP cascade is critical for behavioral plasticity such as learning and memory (Keene and Waddell, 2007), for activity-dependent synaptic plasticity (Zhong and Wu, 1991, Davis et al., 1996, Schuster et al., 1996b, Renger et al., 2000), and for regulation of Ca^{2+} and K^+ currents in neurons and muscles (Zhong and Wu, 1993, Zhao and Wu, 1997, Bhattacharya et al., 1999, Ueda and Wu, 2003). In addition, hyperexcitability-induced synaptic overgrowth by K^+ channel mutations (Budnik et al., 1990, Zhong et al., 1992) is modified by mutations in cAMP metabolism (Zhong et al., 1992, Zhong and Wu, 2004), suppressed by *rut*, but enhanced by increased cAMP levels by *dunce* mutations affecting cAMP-specific phosphodiesterase (Dudai et al., 1976, Byers et al., 1981).

The functional link between cAMP-dependent pathways and K^+ channel-related synaptic plasticity rests on the activity-dependent accumulation of Ca^{2+} , an important regulator of *rut* AC activity (Dudai and Zvi, 1984, Livingstone et al., 1984). Slo channels are colocalized with voltage-activated Ca^{2+} channels and provide a negative feedback to regulate Ca^{2+} -activated events (Salkoff et al., 2006). Thus, enhanced Ca^{2+} influx during development due to the lack of Slo-related negative feedback may trigger a cascade of cAMP-dependent events, leading to pre- and post-synaptic homeostatic modifications observed in *slo* mutants (Fig. 8B).

Such *rut* AC-dependent homeostatic mechanisms may involve downstream effectors, including cAMP-dependent protein kinase (PKA) for protein phosphorylation and cAMP response element-binding protein (CREB) for gene expression (Nguyen and Woo, 2003). PKA-dependent regulation of Sh and Slo currents has been demonstrated in *Drosophila* neurons and muscles (Zhong and Wu, 1993, Esguerra et al., 1994, Yao and Wu, 2001). In addition, elevated PKA activity at larval NMJs reduces quantal size, presumably through modulation of glutamate receptor channel (DGluRIIA) (Davis et al., 1998). However, since acute blockade of Slo current by ChTx treatment does not initiate homeostatic processes (see above), long-term homeostatic modifications observed in *slo* mutants likely involve CREB-dependent gene expression.

Future studies in synaptic homeostasis

It should be noted that *slo*-induced modifications are not fully prevented in *rut;;slo* double mutants. Their postsynaptic receptor composition and associated quantal size are still not identical to WT, albeit significantly different from those in *slo* (Fig. 7C), suggesting potential involvement of other Ca^{2+} -dependent second-messenger cascades in homeostasis. It will be interesting to examine whether Ca^{2+} /CaM-dependent kinase II, known to modulate K^+ currents (Yao and Wu, 2001), activity-dependent synaptic plasticity (Wang et al., 1994,

Park et al., 2002), and behavioral plasticity (Griffith et al., 1993, Jin et al., 1998) in *Drosophila*, also participates in synaptic homeostasis.

Our results highlight the significance of Ca^{2+} influx regulated by Slo K^+ channels in homeostasis. It will be important to determine the source of Ca^{2+} influx that triggers compensatory pre- and post-synaptic modifications. Two Ca^{2+} channel genes, *cacophony* (*cac*) (Smith et al., 1996, Kawasaki et al., 2000, Rieckhof et al., 2003, Peng and Wu, 2007) and *Dmca1D* for the major neuronal and muscular Ca^{2+} channels (Gielow et al., 1995, Zheng et al., 1995), respectively, have been identified in *Drosophila*. Reduced Slo current associated with compensatory increase in Sh I_A in cultured *cac* neurons (Peng and Wu, 2007) suggests a similar contribution of *cac* channels in presynaptic homeostasis. It will be interesting to ask whether *Dmca1D*-dependent Ca^{2+} influx contributes to postsynaptic homeostatic mechanisms through functional coupling with Slo channels.

The diversity of K^+ channels, characterized by distinct gating, activation and inactivation properties (Armstrong, 2003), may lead to different profiles of Ca^{2+} influx in subcellular regions upon their dysfunction (Berke et al., 2006). Therefore, it will be interesting to see whether mutations in other K^+ channels can trigger homeostatic adjustments different from those observed in *slo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NMJs	neuromuscular junctions
<i>Sh</i> (Sh)	<i>Shaker</i> (Shaker)
<i>slo</i> (Slo)	<i>slowpoke</i> (Slowpoke)
<i>rut</i>	<i>rutabaga</i>
AC	Adenylyl cyclase
IIA	DGluRIIA
IIB	DGluRIIB
V	ventral
D	dorsal
M	muscle(s)
EJP	excitatory junctional potential
mEJP	miniature excitatory junctional potential
I_A	transient A-type K^+ current
$\text{I}_{K(\text{Ca})}$	large conductance, Ca^{2+} -activated K^+ current
4-AP	4-aminopyridine

ChTx Charybdotoxin

References

- Armstrong CM. Voltage-gated K channels. *Sci STKE*. 2003; 2003:re10. [PubMed: 12824476]
- Atkinson NS, Robertson GA, Ganetzky B. A component of calcium-activated potassium channels encoded by the *Drosophila slo* locus. *Science*. 1991; 253:551–555. [PubMed: 1857984]
- Atwood HL, Govind CK, Wu CF. Differential ultrastructure of synaptic terminals on ventral longitudinal abdominal muscles in *Drosophila* larvae. *J Neurobiol*. 1993; 24:1008–1024. [PubMed: 8409966]
- Barnes-Davies M, Barker MC, Osmani F, Forsythe ID. Kv1 currents mediate a gradient of principal neuron excitability across the tonotopic axis in the rat lateral superior olive. *Eur J Neurosci*. 2004; 19:325–333. [PubMed: 14725627]
- Bastiani MJ, Harrelson AL, Snow PM, Goodman CS. Expression of fasciclin I and II glycoproteins on subsets of axon pathways during neuronal development in the grasshopper. *Cell*. 1987; 48:745–755. [PubMed: 3545496]
- Berke BA, Lee J, Peng IF, Wu CF. Sub-cellular Ca^{2+} dynamics affected by voltage- and Ca^{2+} -gated K^{+} channels: regulation of the soma-growth cone disparity and the quiescent state in *drosophila* neurons. *Neuroscience*. 2006
- Bhattacharya A, Gu GG, Singh S. Modulation of dihydropyridine-sensitive calcium channels in *Drosophila* by a cAMP-mediated pathway. *J Neurobiol*. 1999; 39:491–500. [PubMed: 10380071]
- Budnik V, Koh YH, Guan B, Hartmann B, Hough C, Woods D, Gorczyca M. Regulation of synapse structure and function by the *Drosophila* tumor suppressor gene *dlg*. *Neuron*. 1996; 17:627–640. [PubMed: 8893021]
- Budnik V, Zhong Y, Wu CF. Morphological plasticity of motor axons in *Drosophila* mutants with altered excitability. *J Neurosci*. 1990; 10:3754–3768. [PubMed: 1700086]
- Byers D, Davis RL, Kiger JA Jr. Defect in cyclic AMP phosphodiesterase due to the *dunce* mutation of learning in *Drosophila melanogaster*. *Nature*. 1981; 289:79–81. [PubMed: 6256649]
- Covarrubias M, Wei AA, Salkoff L. Shaker, Shal, Shab, and Shaw express independent K^{+} current systems. *Neuron*. 1991; 7:763–773. [PubMed: 1742024]
- Davis GW, DiAntonio A, Petersen SA, Goodman CS. Postsynaptic PKA controls quantal size and reveals a retrograde signal that regulates presynaptic transmitter release in *Drosophila*. *Neuron*. 1998; 20:305–315. [PubMed: 9491991]
- Davis GW, Schuster CM, Goodman CS. Genetic dissection of structural and functional components of synaptic plasticity. III. CREB is necessary for presynaptic functional plasticity. *Neuron*. 1996; 17:669–679. [PubMed: 8893024]
- DiAntonio A, Petersen SA, Heckmann M, Goodman CS. Glutamate receptor expression regulates quantal size and quantal content at the *Drosophila* neuromuscular junction. *J Neurosci*. 1999; 19:3023–3032. [PubMed: 10191319]
- Dudai Y, Jan YN, Byers D, Quinn WG, Benzer S. *dunce*, a mutant of *Drosophila* deficient in learning. *Proc Natl Acad Sci U S A*. 1976; 73:1684–1688. [PubMed: 818641]
- Dudai Y, Uzzan A, Zvi S. Abnormal activity of adenylate cyclase in the *Drosophila* memory mutant *rutabaga*. *Neurosci Lett*. 1983; 42:207–212. [PubMed: 6420732]
- Dudai Y, Zvi S. Adenylate cyclase in the *Drosophila* memory mutant *rutabaga* displays an altered Ca^{2+} sensitivity. *Neurosci Lett*. 1984; 47:119–124. [PubMed: 6462535]
- Esguerra M, Wang J, Foster CD, Adelman JP, North RA, Levitan IB. Cloned Ca^{2+} -dependent K^{+} channel modulated by a functionally associated protein kinase. *Nature*. 1994; 369:563–565. [PubMed: 8202157]
- Etheredge JA, Murchison D, Abbott LC, Griffith WH. Functional compensation by other voltage-gated Ca^{2+} channels in mouse basal forebrain neurons with $Ca(V)2.1$ mutations. *Brain Res*. 2007; 1140:105–119. [PubMed: 16364258]

- Featherstone DE, Rushton E, Rohrbough J, Liebl F, Karr J, Sheng Q, Rodesch CK, Broadie K. An essential *Drosophila* glutamate receptor subunit that functions in both central neuropil and neuromuscular junction. *J Neurosci*. 2005; 25:3199–3208. [PubMed: 15788777]
- Feng Y, Ueda A, Wu CF. A modified minimal hemolymph-like solution, HL3.1, for physiological recordings at the neuromuscular junctions of normal and mutant *Drosophila* larvae. *J Neurogenet*. 2004; 18:377–402. [PubMed: 15763995]
- Ferguson GD, Storm DR. Why calcium-stimulated adenylyl cyclases? *Physiology (Bethesda)*. 2004; 19:271–276. [PubMed: 15381755]
- Fettiplace R, Fuchs PA. Mechanisms of hair cell tuning. *Annu Rev Physiol*. 1999; 61:809–834. [PubMed: 10099711]
- Fukui I, Ohmori H. Tonotopic gradients of membrane and synaptic properties for neurons of the chicken nucleus magnocellularis. *J Neurosci*. 2004; 24:7514–7523. [PubMed: 15329398]
- Ganetzky B, Wu CF. Neurogenetic analysis of potassium currents in *Drosophila*: synergistic effects on neuromuscular transmission in double mutants. *J Neurogenet*. 1983; 1:17–28. [PubMed: 6100303]
- Gho M, Ganetzky B. Analysis of repolarization of presynaptic motor terminals in *Drosophila* larvae using potassium-channel-blocking drugs and mutations. *J Exp Biol*. 1992; 170:93–111. [PubMed: 1328458]
- Gielow ML, Gu GG, Singh S. Resolution and pharmacological analysis of the voltage-dependent calcium channels of *Drosophila* larval muscles. *J Neurosci*. 1995; 15:6085–6093. [PubMed: 7666192]
- Griffith LC, Verselis LM, Aitken KM, Kyriacou CP, Danho W, Greenspan RJ. Inhibition of calcium/calmodulin-dependent protein kinase in *Drosophila* disrupts behavioral plasticity. *Neuron*. 1993; 10:501–509. [PubMed: 8384859]
- Jan LY, Jan YN. Antibodies to horseradish peroxidase as specific neuronal markers in *Drosophila* and in grasshopper embryos. *Proc Natl Acad Sci U S A*. 1982; 79:2700–2704. [PubMed: 6806816]
- Jan YN, Jan LY, Dennis MJ. Two mutations of synaptic transmission in *Drosophila*. *Proc R Soc Lond B Biol Sci*. 1977; 198:87–108. [PubMed: 20636]
- Jin P, Griffith LC, Murphey RK. Presynaptic calcium/calmodulin-dependent protein kinase II regulates habituation of a simple reflex in adult *Drosophila*. *J Neurosci*. 1998; 18:8955–8964. [PubMed: 9787001]
- Johansen J, Halpern ME, Johansen KM, Keshishian H. Stereotypic morphology of glutamatergic synapses on identified muscle cells of *Drosophila* larvae. *J Neurosci*. 1989; 9:710–725. [PubMed: 2563766]
- Jonas EA, Kaczmarek LK. Regulation of potassium channels by protein kinases. *Curr Opin Neurobiol*. 1996; 6:318–323. [PubMed: 8794088]
- Kamb A, Iverson LE, Tanouye MA. Molecular characterization of Shaker, a *Drosophila* gene that encodes a potassium channel. *Cell*. 1987; 50:405–413. [PubMed: 2440582]
- Kawasaki F, Felling R, Ordway RW. A temperature-sensitive paralytic mutant defines a primary synaptic calcium channel in *Drosophila*. *J Neurosci*. 2000; 20:4885–4889. [PubMed: 10864946]
- Keene AC, Waddell S. *Drosophila* olfactory memory: single genes to complex neural circuits. *Nat Rev Neurosci*. 2007; 8:341–354. [PubMed: 17453015]
- Lahey T, Gorczyca M, Jia XX, Budnik V. The *Drosophila* tumor suppressor gene *dlg* is required for normal synaptic bouton structure. *Neuron*. 1994; 13:823–835. [PubMed: 7946331]
- Landgraf M, Bossing T, Technau GM, Bate M. The origin, location, and projections of the embryonic abdominal motoneurons of *Drosophila*. *J Neurosci*. 1997; 17:9642–9655. [PubMed: 9391019]
- Livingstone MS, Sziber PP, Quinn WG. Loss of calcium/calmodulin responsiveness in adenylate cyclase of rutabaga, a *Drosophila* learning mutant. *Cell*. 1984; 37:205–215. [PubMed: 6327051]
- MacLean JN, Zhang Y, Johnson BR, Harris-Warrick RM. Activity-independent homeostasis in rhythmically active neurons. *Neuron*. 2003; 37:109–120. [PubMed: 12526777]
- Marder E, Goaillard JM. Variability, compensation and homeostasis in neuron and network function. *Nat Rev Neurosci*. 2006; 7:563–574. [PubMed: 16791145]
- Marrion NV, Tavalin SJ. Selective activation of Ca²⁺-activated K⁺ channels by co-localized Ca²⁺ channels in hippocampal neurons. *Nature*. 1998; 395:900–905. [PubMed: 9804423]

- Marrus SB, Portman SL, Allen MJ, Moffat KG, DiAntonio A. Differential localization of glutamate receptor subunits at the *Drosophila* neuromuscular junction. *J Neurosci*. 2004; 24:1406–1415. [PubMed: 14960613]
- Martin AR. A further study of the statistical composition on the end-plate potential. *Journal of Physiology*. 1955; 130:114–122. [PubMed: 13278890]
- Nguyen PV, Woo NH. Regulation of hippocampal synaptic plasticity by cyclic AMP-dependent protein kinases. *Prog Neurobiol*. 2003; 71:401–437. [PubMed: 15013227]
- O'Brien RJ, Kamboj S, Ehlers MD, Rosen KR, Fischbach GD, Huganir RL. Activity-dependent modulation of synaptic AMPA receptor accumulation. *Neuron*. 1998; 21:1067–1078. [PubMed: 9856462]
- Papazian DM, Schwarz TL, Tempel BL, Jan YN, Jan LY. Cloning of genomic and complementary DNA from Shaker, a putative potassium channel gene from *Drosophila*. *Science*. 1987; 237:749–753. [PubMed: 2441470]
- Paradis S, Sweeney ST, Davis GW. Homeostatic control of presynaptic release is triggered by postsynaptic membrane depolarization. *Neuron*. 2001; 30:737–749. [PubMed: 11430807]
- Park D, Coleman MJ, Hodge JJ, Budnik V, Griffith LC. Regulation of neuronal excitability in *Drosophila* by constitutively active CaMKII. *J Neurobiol*. 2002; 52:24–42. [PubMed: 12115891]
- Peng IF, Wu CF. *Drosophila* cacophony channels: a major mediator of neuronal Ca²⁺ currents and a trigger for K⁺ channel homeostatic regulation. *J Neurosci*. 2007; 27:1072–1081. [PubMed: 17267561]
- Petersen SA, Fetter RD, Noordermeer JN, Goodman CS, DiAntonio A. Genetic analysis of glutamate receptors in *Drosophila* reveals a retrograde signal regulating presynaptic transmitter release. *Neuron*. 1997; 19:1237–1248. [PubMed: 9427247]
- Qin G, Schwarz T, Kittel RJ, Schmid A, Rasse TM, Kappei D, Ponimaskin E, Heckmann M, Sigrist SJ. Four different subunits are essential for expressing the synaptic glutamate receptor at neuromuscular junctions of *Drosophila*. *J Neurosci*. 2005; 25:3209–3218. [PubMed: 15788778]
- Renger JJ, Ueda A, Atwood HL, Govind CK, Wu CF. Role of cAMP cascade in synaptic stability and plasticity: ultrastructural and physiological analyses of individual synaptic boutons in *Drosophila* memory mutants. *J Neurosci*. 2000; 20:3980–3992. [PubMed: 10818133]
- Rieckhof GE, Yoshihara M, Guan Z, Littleton JT. Presynaptic N-type calcium channels regulate synaptic growth. *J Biol Chem*. 2003; 278:41099–41108. [PubMed: 12896973]
- Robitaille R, Garcia ML, Kaczorowski GJ, Charlton MP. Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release. *Neuron*. 1993; 11:645–655. [PubMed: 7691106]
- Salkoff L, Butler A, Ferreira G, Santi C, Wei A. High-conductance potassium channels of the SLO family. *Nat Rev Neurosci*. 2006; 7:921–931. [PubMed: 17115074]
- Schuster CM, Davis GW, Fetter RD, Goodman CS. Genetic dissection of structural and functional components of synaptic plasticity. I. Fasciclin II controls synaptic stabilization and growth. *Neuron*. 1996a; 17:641–654. [PubMed: 8893022]
- Schuster CM, Davis GW, Fetter RD, Goodman CS. Genetic dissection of structural and functional components of synaptic plasticity. II. Fasciclin II controls presynaptic structural plasticity. *Neuron*. 1996b; 17:655–667. [PubMed: 8893023]
- Schuster CM, Ultsch A, Schloss P, Cox JA, Schmitt B, Betz H. Molecular cloning of an invertebrate glutamate receptor subunit expressed in *Drosophila* muscle. *Science*. 1991; 254:112–114. [PubMed: 1681587]
- Sigrist SJ, Reiff DF, Thiel PR, Steinert JR, Schuster CM. Experience-dependent strengthening of *Drosophila* neuromuscular junctions. *J Neurosci*. 2003; 23:6546–6556. [PubMed: 12878696]
- Smith LA, Wang X, Peixoto AA, Neumann EK, Hall LM, Hall JC. A *Drosophila* calcium channel alpha1 subunit gene maps to a genetic locus associated with behavioral and visual defects. *J Neurosci*. 1996; 16:7868–7879. [PubMed: 8987815]
- Stewart BA, Atwood HL, Renger JJ, Wang J, Wu CF. Improved stability of *Drosophila* larval neuromuscular preparations in haemolymph-like physiological solutions. *J Comp Physiol [A]*. 1994; 175:179–191.

- Stewart BA, Schuster CM, Goodman CS, Atwood HL. Homeostasis of synaptic transmission in *Drosophila* with genetically altered nerve terminal morphology. *J Neurosci*. 1996; 16:3877–3886. [PubMed: 8656281]
- Tanouye MA, Ferrus A. Action potentials in normal and Shaker mutant *Drosophila*. *J Neurogenet*. 1985; 2:253–271. [PubMed: 3936906]
- Tanouye MA, Ferrus A, Fujita SC. Abnormal action potentials associated with the Shaker complex locus of *Drosophila*. *Proc Natl Acad Sci U S A*. 1981; 78:6548–6552. [PubMed: 16593105]
- Tejedor FJ, Bokhari A, Rogero O, Gorczyca M, Zhang J, Kim E, Sheng M, Budnik V. Essential role for *dlg* in synaptic clustering of Shaker K⁺ channels in vivo. *J Neurosci*. 1997; 17:152–159. [PubMed: 8987744]
- Thomas U, Kim E, Kuhlendahl S, Koh YH, Gundelfinger ED, Sheng M, Garner CC, Budnik V. Synaptic clustering of the cell adhesion molecule fasciclin II by discs-large and its role in the regulation of presynaptic structure. *Neuron*. 1997; 19:787–799. [PubMed: 9354326]
- Turrigiano GG, Leslie KR, Desai NS, Rutherford LC, Nelson SB. Activity-dependent scaling of quantal amplitude in neocortical neurons. *Nature*. 1998; 391:892–896. [PubMed: 9495341]
- Ueda, A.; Wu, CF. REDUCED SYNAPTIC STRENGTH AND INCREASED VARIABILITY OF AXONAL TERMINAL BRANCHES IN DROSOPHILA MEMORY MUTANTS: PRESYNAPTIC IONIC MECHANISMS. Program No 45712 2003 Abstract Viewer/Itinerary Planner; 2003; Washington, DC: Society for Neuroscience; 2003. Online
- Ueda A, Wu CF. Distinct frequency-dependent regulation of nerve terminal excitability and synaptic transmission by IA and IK potassium channels revealed by *Drosophila* Shaker and Shab mutations. *J Neurosci*. 2006; 26:6238–6248. [PubMed: 16763031]
- Wang J, Renger JJ, Griffith LC, Greenspan RJ, Wu CF. Concomitant alterations of physiological and developmental plasticity in *Drosophila* CaM kinase II-inhibited synapses. *Neuron*. 1994; 13:1373–1384. [PubMed: 7993628]
- Warbington L, Hillman T, Adams C, Stern M. Reduced transmitter release conferred by mutations in the slowpoke-encoded Ca²⁺-activated K⁺ channel gene of *Drosophila*. *Invert Neurosci*. 1996; 2:51–60. [PubMed: 9372155]
- Wu CF, Ganetzky B, Haugland FN, Liu AX. Potassium currents in *Drosophila*: different components affected by mutations of two genes. *Science*. 1983; 220:1076–1078. [PubMed: 6302847]
- Yao WD, Wu CF. Distinct roles of CaMKII and PKA in regulation of firing patterns and K⁺ currents in *Drosophila* neurons. *J Neurophysiol*. 2001; 85:1384–1394. [PubMed: 11287463]
- Yazegian B, DiGregorio DA, Vergara JL, Poage RE, Meriney SD, Grinnell AD. Direct measurements of presynaptic calcium and calcium-activated potassium currents regulating neurotransmitter release at cultured *Xenopus* nerve-muscle synapses. *J Neurosci*. 1997; 17:2990–3001. [PubMed: 9096135]
- Zhao ML, Wu CF. Alterations in frequency coding and activity dependence of excitability in cultured neurons of *Drosophila* memory mutants. *J Neurosci*. 1997; 17:2187–2199. [PubMed: 9045743]
- Zheng W, Feng G, Ren D, Eberl DF, Hannan F, Dubald M, Hall LM. Cloning and characterization of a calcium channel $\alpha 1$ subunit from *Drosophila melanogaster* with similarity to the rat brain type D isoform. *J Neurosci*. 1995; 15:1132–1143. [PubMed: 7869089]
- Zhong Y, Budnik V, Wu CF. Synaptic plasticity in *Drosophila* memory and hyperexcitable mutants: role of cAMP cascade. *J Neurosci*. 1992; 12:644–651. [PubMed: 1371316]
- Zhong Y, Wu CF. Altered synaptic plasticity in *Drosophila* memory mutants with a defective cyclic AMP cascade. *Science*. 1991; 251:198–201. [PubMed: 1670967]
- Zhong Y, Wu CF. Differential modulation of potassium currents by cAMP and its long-term and short-term effects: *dunce* and *rutabaga* mutants of *Drosophila*. *J Neurogenet*. 1993; 9:15–27. [PubMed: 8295075]
- Zhong Y, Wu CF. Neuronal activity and adenylyl cyclase in environment-dependent plasticity of axonal outgrowth in *Drosophila*. *J Neurosci*. 2004; 24:1439–1445. [PubMed: 14960616]
- Zito K, Fetter RD, Goodman CS, Isacoff EY. Synaptic clustering of Fascilin II and Shaker: essential targeting sequences and role of *Dlg*. *Neuron*. 1997; 19:1007–1016. [PubMed: 9390515]

Fig. 1. Naturally occurring dorsal-ventral disparity in excitatory junctional potential (EJP) amplitudes correlated with differential regulation of presynaptic neurotransmitter release, postsynaptic receptor composition, and mEJP amplitudes

(A) Larval body-wall musculature within a single hemisegment from the ventral (V) to dorsal (D) midline of a third instar larva, with neuromuscular junctions (NMJs, isomorphic) on muscles 6, 4, and 1 and corresponding representative EJPs. The numbering system for the identified muscles is shown. (B) Pooled EJP amplitude data from muscles in the abdominal hemisegments are shown in the box plots together with Mean $\pm$ SEM values with respect to their locations along the D-V axis. For each larva, 5 to 8 muscles in a single hemisegment are sampled. The total number of muscle fibers is indicated (equivalent to the number of larvae except for muscles 6/7, 19 muscle fibers from 13 larvae). The box plot in this and following figures represents 25 through 75% with median and 5 and 95% indicated by bars. (C) Two representative traces of spontaneous mEJPs in WT are shown for ventral (M6/7) and dorsal (M1/9) NMJs. Scale bar, 1 mV and 100 ms. (D) Average quantal size (mEJP amplitude) (top) and quantal content (bottom) at ventral (M6/7) and dorsal (M1/9) NMJs in WT. Note a significantly smaller quantal size and quantal content at dorsal NMJs. *, $P < 0.05$ and **, $P < 0.01$, t -test for ventral vs. dorsal NMJs. (E) Relative ratio of DGluRIIA over DGluRIIB (IIA/IIB) immunoreactivity visualized by double-labeling at the same type Ib NMJs reveals gradual changes along the D-V axis, resembling the D-V profile of EJP amplitude shown in (B) (see Experimental procedures). 17 hemisegments in 5 larvae.

Fig. 2. Differential distribution of synaptic proteins between ventral and dorsal NMJs
Immunoreactivity against DGluRIIA (A), DGluRIIB (B), FasII (C), and Dlg (D) is shown for ventral (M6/7) and dorsal (M1) NMJs in WT. The approximate boundary between muscles 6 (bottom) and 7 (top) is indicated by a dashed line in (D). Scale bar, 20 μ m. (E) D-V differences in the density of receptor and other synaptic proteins. The D/V (M1/M6-7) ratio of pixel intensity is shown with the number of NMJs examined (see Experimental procedures for the measurement). Note opposite trends in the ratios of DGluRIIA vs. FasII, and, to a lesser extent, DGluRIIB. ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$, t -test against the null hypothesis of equal D-V intensity (or the ratio of 1.0).

Fig. 3. Homeostatic maintenance of synaptic strength in *slo* and *Sh* mutants and their Ca^{2+} -dependent mutant phenotypes in EJP amplitudes

(A) D-V differences in EJP amplitude from WT, *slo*, and *Sh* in the presence of 0.5 (top) and 0.2 (bottom) mM $[\text{Ca}^{2+}]_o$ in HL3.1 saline. Top traces in the Ca 0.2 panel represent the general trends while those at the bottom depict extreme cases, including the reversed D-V differentials, with larger EJPs at dorsal NMJs in mutants. Scale bar, 10 mV and 25 ms, unless otherwise indicated. (B) Pooled data for D-V differences at a low Ca^{2+} level (0.2 mM). Ventral (V) and dorsal (D) EJP data of the same larva are linked. Note larger EJPs at dorsal NMJs in subsets of *slo* and *Sh* mutants (lines with positive slopes). Number of larvae: 25 for WT, 22 for *slo*, and 14 for *Sh*. Open and closed symbols, Mean $\pm$ SEM before and after the correction procedure for non-linear summation, respectively (see Experimental procedures). ***, $P < 0.001$, t -test for ventral vs. dorsal NMJs. (C) Histogram for the EJP ratio (V/D) in linear and logarithmic scales (see Experimental procedures). Vertical line in each plot distinguishes the normal (right) and reversed (left) D-V differences. Note cases with reversed differences in *slo* and *Sh*. Mean (indicated by arrows) and SEM in parentheses.

Fig. 4. Altered postsynaptic receptor composition and synaptic protein density in *slo* mutants
 Immunoreactivity against FasII (A₁ and A₂), DGluRIIA (B₁ and B₂), and Dlg (C₁ and C₂) in *slo* and *Sh* mutants is shown for both ventral (M6/7) and dorsal (M1) NMJs. (D) Ratio of pixel intensity (M1/M6-7) among WT, *slo*, and *Sh*. Note a significant difference in the FasII, DGluRIIA, and Dlg ratios in *slo* compared with WT and/or *Sh*. The number of NMJs examined in WT, *slo*, and *Sh*: 13, 18, and 8 for FasII; 40, 28, and 21 for DGluRIIA; 30, 18, and 17 for DGluRIIB; 25, 12, and 4 for DGluRIIC; 25, 18, and 6 for Dlg; 47, 27, and 35 for HRP. WT data from Fig. 3 are shown as reference. (E) Decrease in the DGluRIIA/IIB (IIA/IIB) ratio in *slo* NMJs. The IIA/IIB ratio from DGluRIIA and DGluRIIB double staining experiments is normalized to that in WT ventral NMJs (dashed lines). The number of ventral

and dorsal NMJs examined: 15 and 15 for WT, 20 and 19 for *slo*, and 20 and 20 for *Sh*. Mean $\pm$ SD in D and E. ***, $P < 0.001$, *, $P < 0.05$, one-way ANOVA.

Fig. 5. Modified quantal size and quantal content in *slo* and *Sh* mutants

(A) Two sample traces of mEJPs recorded from ventral (M6/7) and dorsal (M1/9) NMJs of each genotype. (B) Pooled data of quantal size (top) and quantal content (bottom) with the number of larvae examined indicated. Note a significant reduction in quantal size at *slo* ventral NMJs and drastic increase in *Sh* quantal content. WT data in Fig. 1D are shown as reference. Mean $\pm$ SD; ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$, one-way ANOVA among genotypes. +, $P < 0.05$, ++, $P < 0.01$, t -test for ventral vs. dorsal NMJs within a genotype.

Fig. 6. Contribution of Sh current in the natural D-V disparity in synaptic strength and its homeostatic upregulation in *slo* mutants

(A) Proportionally greater increase in EJP amplitude induced by 4-AP treatment at dorsal than ventral NMJs in WT. As shown in the ensemble plot (right), the EJP V/D ratios are reduced after 4-AP treatment in nearly all samples, as indicated by gray circles below the 45-degree diagonal line. (B) Greater EJP increase (Δ EJP) in *slo* after sequential application of 4-AP with increasing concentrations at ventral NMJs. The number of larvae examined, WT vs. *slo* at [4-AP]: 8 vs. 6 at both 0.02 and 0.1; 6 vs. 5 at 0.2; 2 vs. 3 at 1.0 mM. ***, $P < 0.001$, **, $P < 0.01$, t -test between WT and *slo*. (C) Two representative examples of EJP responses demonstrating presynaptic hyperexcitability caused by suppression of both Slo and Sh currents upon mutational or pharmacological (4-AP and ChTx) manipulations. Scale bar, 10 mV and 200 ms for the top row, and 10 mV and 100 ms for the bottom row, unless otherwise indicated.

Fig. 7. Actions of *rut* AC in homeostatic regulation of synaptic strength in *slo* mutants through modifications of postsynaptic receptor composition, quantal size, and 4-AP-sensitive Sh I_A
 (A) Representative traces of EJPs at 0.2 mM $[\text{Ca}^{2+}]_o$ for WT, *slo*, *rut*, and *rut;;slo*. Note similar EJP sizes between WT and *rut;;slo*, in contrast to smaller sizes in both *slo* and *rut* mutants. (B) Pooled EJP amplitudes summarized in box plots. The number of larvae examined in WT, *rut*, *slo*, and *rut;;slo*: 25, 15, 22, and 19 for ventral; 25, 10, 22, and 11 for dorsal NMJs. (C) Increases in IIA/IIB ratio and quantal size at ventral NMJs in *rut;;slo* that are partially restored to WT values. *rut;;slo* double mutants significantly differ from *slo* in postsynaptic receptor composition (IIA/IIB) (left) and correlated quantal size (right). (D) Quantal content decrease in *rut* and enhancement in *rut;;slo* double mutants at ventral NMJs.

(E) EJP increase at ventral NMJs upon 4-AP treatment (0.2 mM) in *rut*, *slo*, and *rut;;slo*. Mean $\pm$ SEM with numbers of NMJs (IIA/IIB ratio) and larvae (quantal size, quantal content, and Δ EJP). EJP amplitudes for (B), (D), and (E) are corrected for non-linear summation (see Experimental procedures). Data shown in (C) and (D) are normalized to WT values indicated by dashed lines. ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$, t -test (B–D) and one-way ANOVA (Δ EJP in (E)).

A D-V Gradient (WT, Ca 0.2 mM)

● Glu Release DGluRIIA DGluRIIB

B Homeostasis (Ventral, Ca 0.5 mM)

Fig. 8. Roles of Slo and Sh K^+ channels in the natural variation in synaptic strength along the D-V axis and cAMP-dependent synaptic homeostasis

(A) Plausible pre- and post-synaptic mechanisms underlying the D-V disparity in synaptic strength. Note the increased number of Sh channels correlated with reduced number of vesicle release and more abundant DGluRIIB subunits associated with smaller mEJP amplitude at dorsal (right) compared to ventral (left) NMJs. (B) As Ca^{2+} influx increases at higher $[Ca^{2+}]_o$ (0.5 mM, dark blue), full-blown Slo current (dark blue) plays a major role in repolarizing membrane and controlling vesicle release (left). In *slo* mutants (right), the loss of a negative feedback onto Ca^{2+} influx provided by Slo current may lead to an increase in $[Ca^{2+}]_i$ (**bold**), which, in turn, may initiate *rut* AC/cAMP-dependent presynaptic upregulation of Sh current, compensating in part for the loss of Slo current (note enhanced releases in *slo*). Concomitant postsynaptic decrease in IIA/IIB ratio and mEJP amplitude via *rut* regulation leads to further restoration of synaptic strength (note similar EJPs between WT and *slo* as the ensemble quantal events in top traces). However, with less Slo current activation at lower $[Ca^{2+}]_o$ (0.2 mM, light blue), Sh I_A plays a dominant role in regulating membrane repolarization and vesicle release. A combination of upregulated presynaptic Sh current and reduced IIA/IIB ratio and mEJP amplitude would then lead to a smaller EJP in *slo* mutants (bottom traces).