Slob, a Slowpoke channel–binding protein, modulates synaptic transmission

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Modulation of ion channels by regulatory proteins within the same macromolecular complex is a well-accepted concept, but the physiological consequences of such modulation are not fully understood. Slowpoke (Slo), a potassium channel critical for action potential repolarization and transmitter release, is regulated by Slo channel–binding protein (Slob), a *Drosophila melanogaster* Slo (dSlo) binding partner. Slob modulates the voltage dependence of dSlo channel activation in vitro and exerts similar effects on the dSlo channel in *Drosophila* central nervous system neurons in vivo. In addition, Slob modulates action potential duration in these neurons. Here, we investigate further the functional consequences of the modulation of the dSlo channel by Slob in vivo, by examining larval neuromuscular synaptic transmission in flies in which Slob levels have been altered. In Slob-null flies generated through P-element mutagenesis, as well as in Slob knockdown flies generated by RNA interference (RNAi), we find an enhancement of synaptic transmission but no change in the properties of the postsynaptic muscle cell. Using targeted transgenic rescue and targeted expression of Slob-RNAi, we find that Slob expression in neurons (but not in the postsynaptic muscle cell) is critical for its effects on synaptic transmission. Furthermore, inhibition of dSlo channel activity abolishes these effects of Slob. These results suggest that presynaptic Slob, by regulating dSlo channel function, participates in the modulation of synaptic transmission.

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INTRODUCTION

Slowpoke (Slo) is a large-conductance voltage-gated, calcium-dependent potassium channel (Atkinson et al., 1991; Adelman et al., 1992; Tseng-Crank et al., 1994). It is involved in a variety of physiological phenomena, including the regulation of cell excitability, neurotransmitter release, and muscle contraction (Elkins and Ganetzky, 1988; Singh and Wu, 1990; Warbington et al., 1996; Atkinson et al., 2000). Slo is often associated with auxiliary subunits that interact with the channel and modulate its activity (Lu et al., 2006). For example, mammalian Slo channels bind to multiple distinct β subunits, each of which modulates channel function in different ways (Weiger et al., 2002). The Slo channel–binding protein (Slob) was discovered in a yeast two-hybrid screen using the C-terminal tail region of the *Drosophila melanogaster* Slo (dSlo) calcium-dependent potassium channel as bait (Schopperle et al., 1998). Multiple Slob variants arise from alternative splicing and multiple translational start sites; these Slob variants are named based on their molecular weights (in kilodaltons), Slob51, 57, 65, and 71 (Jaramillo et al., 2006).

Using patch recordings from cells cotransfected with dSlo and different Slob variants to investigate the specific effects of each Slob on dSlo channel function, we found that Slob57 (the most prominent Slob isoform) and Slob51 shift the dSlo conductance–voltage relationship to more depolarized voltages as well as lead to channel inactivation and faster deactivation of dSlo. The other Slob variants shift the conductance–voltage relationship of dSlo to less depolarized voltages and have no effect on dSlo kinetics (Zeng et al., 2005). The amino-terminal region of the Slob variants appears to be critical in determining their specific effects on dSlo (Zeng et al., 2005).

Slob mRNA and protein are expressed in many areas of the *Drosophila* brain, including pars intercerebralis (PI) neurons, photoreceptors, and the optic lobe (Jaramillo et al., 2004). Slob protein is also expressed at the larval neuromuscular junction (NMJ) (Zhou et al., 1999). Slob is expressed especially prominently in the PI neurons (Jaramillo et al., 2004), and patch recordings from these neurons in vivo reveal a role for Slob in the modulation of neuronal dSlo channels and action potential duration (Shahidullah et al., 2009).

Slob colocalizes with dSlo as well as with another signaling protein, 14-3-3, at the presynaptic terminal of the NMJ (Zhou et al., 1999). In the current study, we examined the function of Slob in synaptic transmission at the larval NMJ, using a combination of genetic manipulation and voltage clamp recording techniques. Knockout

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Abbreviations used in this paper: dSlo, *Drosophila* Slowpoke; EJC, excitatory junctional current; HRP, horseradish peroxidase; mEJC, miniature EJC; NMJ, neuromuscular junction; PI, pars intercerebralis; RNAi, RNA interference; Slo, Slowpoke; Slob, Slo channel–binding protein; TTX, tetrodotoxin; UAS, upstream activation sequence; WT, wild type.

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of Slob by P-element mutagenesis, or knockdown by transgenic expression of Slob-RNAi, leads to increases in the evoked excitatory junctional current (EJC) and higher spontaneous transmitter release. The altered synaptic transmission can be induced by disruption of Slob presynaptically and rescued when Slob expression is restored presynaptically; disruption or restoration of Slob only in postsynaptic muscle cells has no effect. Furthermore, despite the increases in synaptic transmission, muscle cell input resistance and capacitance do not change, indicating that Slob ablation does not change general muscle cell properties. Disruption of the dSlo channel by either pharmacological or genetic manipulation abolishes the effects of altering Slob expression, suggesting that Slob affects synaptic transmission through its modulation of the dSlo channel.

MATERIALS AND METHODS

Drosophila stock

Flies were reared at 25°C on standard *Drosophila* medium. Slob-null lines generated via P-element mutagenesis, Slob knockdown lines generated via expression of upstream activation sequence (UAS) fused with Slob RNA interference (RNAi; Slob-RNAi), and fly lines expressing transgenic UAS-Slob57 were as described previously (Shahidullah et al., 2009). Lines P{GawB}1407 (stock no. 8751; expression of UAS downstream gene in nerve) and P{GawB}how[24B] (stock no. 1767; expression of UAS downstream gene in embryonic mesoderm) were purchased from the Bloomington fly stock center. Actin-GAL4/Tm6B (ubiquitous expression of UAS downstream gene) and elav-Gene Switch (expression of UAS downstream gene in the nervous system) lines were provided by A. Sehgal (University of Pennsylvania, Philadelphia, PA). The elav-Gene Switch line expresses a conditional elav-Gal4 protein whose activation requires the presence of RU-486 (mifepristone; Sigma-Aldrich), a synthetic steroid. We diluted RU-486 in ethanol and mixed it into fly food to reach a final RU-486 concentration of 200 µM. To activate elav-Gene Switch, crosses were performed in food vials containing RU-486, and the offspring larvae were tested to confirm the expression of the UAS downstream gene (Osterwalder et al., 2001; Mao et al., 2004).

Slob-null and rescue flies

As described previously (Shahidullah et al., 2009), the mut^{K162} line has a P-element inserted in a downstream exon of the Slob gene, making it Slob null. The mut^{IP1} line is an imprecise excision line, generated from the mut^{K162} line. Because of the imprecise excision, sequences directly adjacent to the P-element were excised together with the P-element. Thus, the Slob gene and expression of all Slob splice variants are disrupted. WT^{P41} line, a precise excision line also generated from the mut^{K162} line, serves as the wild-type (WT) control, except where noted otherwise. For rescue experiments, fly lines expressing UAS-Slob57 were crossed into a Slob-null background (mut^{IP1}), as were the GAL-4 driver lines. Specific Gal4 lines as mentioned above were used to drive Slob rescue in muscle ($mut^{IP1}rescue^{muscle}$), nervous system ($mut^{IP1}rescue^{nerve}$), or ubiquitously ($mut^{IP1}rescue^{all}$).

Slob-RNAi flies

The uncrossed Slob57 RNAi (WT^{1}) and Actin-Gal4 (WT^{2}) flies were used as WT controls for the RNAi experiments. Gal4 driver lines were used to express Slob57 RNAi and thus disrupt Slob in specific locations, including ubiquitous disruption of Slob (Slob-$RNAi^{all}$), only in nerve (Slob-$RNAi^{nerve}$) or only in muscle cells (Slob-$RNAi^{muscle}$). The Slob57 RNAi construct was designed to selectively target the expression of Slob57 and not the other Slob variants (Shahidullah et al., 2009).

Slob antibody

Polyclonal Slob antibody was purified as described previously (Jaramillo et al., 2004), using serum from rabbits immunized with a GST–Slob fusion protein. Specificity of the Slob antibody for immunohistochemical staining of fly tissues was tested previously (Jaramillo et al., 2004).

Western blot

Fly heads (~50) were homogenized in 5 ml of lysis buffer containing 1% CHAPS, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 120 mM NaCl, 50 mM KCl, 2 mM DTT, and 10 µl of protease inhibitor cocktail (Sigma-Aldrich). DC Protein Assay (Bio-Rad Laboratories) was used to measure protein concentration in the lysates. Equal amounts of protein were loaded on a polyacrylamide gel before a final transfer onto nitrocellulose membranes. Blots were blocked with 5% nonfat milk in TBST (0.1% Tween 20 in Tris-buffered saline) and probed with anti-Slob antibody overnight. The blots were washed with TBST before incubation with horseradish peroxidase (HRP)-conjugated donkey anti–rabbit secondary antibody (GE Healthcare) for 1 h. Finally, the signals were detected using the Enhanced Chemiluminescence Detection System (GE Healthcare).

Immunostaining

Fly larvae were dissected at 4°C as for electrophysiological recordings (described below), fixed in 4% paraformaldehyde for 30 min, and blocked with 10% normal donkey serum in PBS containing 0.1% Triton X-100 (PBST) for 1 h. Samples were then incubated overnight at 4°C with rabbit anti-Slob polyclonal antibody (1:1,000) and Texas red–conjugated goat anti-HRP antibody (1:100). Samples were washed in PBST six times for 15 min each before being incubated with the secondary antibodies (FITC-conjugated donkey anti–rabbit IgG and Texas red–conjugated donkey anti–goat IgG; both provided by L. Iacovitti, Thomas Jefferson University, Philadelphia, PA) at a dilution of 1:200 in PBST containing 5% normal donkey serum for 2 h, and washed in PBS six times for 20 min each before being mounted on the slides. Staining was visualized by fluorescence microscopy using a microscope (1X81; Olympus).

Electrophysiological recording from NMJ

Larvae were dissected and recorded in hemolymph-like saline HL3.1 containing (in mM): 70 NaCl, 5 KCl, 4 $MgCl_2$, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, and 5 HEPES, pH 7.2, as described previously (Broadie and Bate, 1993; Feng et al., 2004; Ueda and Wu, 2006). HL3.1 containing 0, 0.1, 0.2, 0.3, 0.6, 1, or 1.8 mM $CaCl_2$ was used for recording. In some experiments, 1 µM tetrodotoxin (TTX) was included in the extracellular solution. The segmental nerves were severed from the ventral ganglion and stimulated with a suction electrode (A-M Systems). Recordings were performed on ventral longitudinal muscles 6, 7, or 13 in abdominal segments A3–A5 of third instar larvae, as described previously (Broadie and Bate, 1993). All cells selected for recording had resting membrane potentials between −50 and −70 mV. Both miniature and evoked postsynaptic currents were recorded while the muscle cell was voltage clamped at −60 mV using an AxoClamp 2A (Axon Instruments) in single-electrode voltage clamp mode (switching frequency, 10 KHz) and sharp microelectrodes (Warner Instruments) filled with 3 M KCl (5–10-MΩ resistance). Groups of data were first tested with one-way ANOVA. When the difference was significant ($P < 0.05$), Student's *t* test was used to

compare the means of two individual groups. The resulting p-values are presented in the text and figures (*, $P < 0.05$; **, $P < 0.01$). Bars in the figures represent the mean ± SEM.

RESULTS

Slob protein expression in Slob-null, Slob-RNAi, and Slob57 rescue flies

We previously made two distinct kinds of Slob deficiency flies: Slob-null flies through P-element mutagenesis, and Slob knockdown flies through RNAi. Among the Slob-null lines, mut^{K162} is a P-element insertion line, and mut^{IP1} is a P-element imprecise excision line obtained by remobilizing the P-element in the mut^{K162} line (Shahidullah et al., 2009). The WT^{P41} line serves as a control for the mut^{IP1} line, as it has an intact Slob gene as a result of precise excision of the P-element. In Slob-RNAi flies, expression of Slob57 RNAi was driven by the recombination of UAS-Slob57 RNAi with specific Gal-4 drivers. Both classes of Slob deficiency flies are viable and do not display any gross anatomical phenotype. All genotypes were confirmed using PCR.

We used Western blot to examine the Slob protein level in the heads of Slob-null, rescue, and Slob-RNAi flies. The mut^{IP1} line shows ablated Slob expression compared with the WT^{P41} flies (Fig. 1 A, lanes 1 and 2). The apparent residual band in the mut^{IP1} flies (Fig. 1 A, lane 2) is nonspecific staining of an unknown protein that is similar in molecular weight but not related to Slob (Jaramillo et al., 2006). Rescue of Slob ubiquitously (Fig. 1 A, lane 3) or in the nervous system (lane 4) restores Slob expression in fly heads. We do not see any changes in dSlo expression in Slob-mutant flies (unpublished data).

In the Slob-RNAi flies (Fig. 1 B), when Slob-RNAi expression is ubiquitous ($Slob\text{-}RNAi^{all}$), Slob protein level is also reduced (lane 2 compared with lane 1). If Slob-RNAi expression is only in muscle (Fig. 1 B, lane 3), Slob protein level in fly heads remains similar to that in WT. Slob-RNAi expression driven by a nerve-specific Gal4 driver does not affect Slob levels in the head (Fig. 1 B, lane 4). The 1407-Gal4 driver drives the expression of the UAS downstream gene in peripheral nerve, peripheral neurons, and a portion of central nervous system neurons (Luo et al., 1994). The fact that we do not see a significant decrease of Slob in fly heads suggests that the expression of Slob-RNAi driven by 1407-Gal4 in the adult fly head is minor.

Manipulating Slob expression in larval muscle and nerve terminals

To determine the effects of various genetic manipulations on Slob expression in motor neurons and muscle,

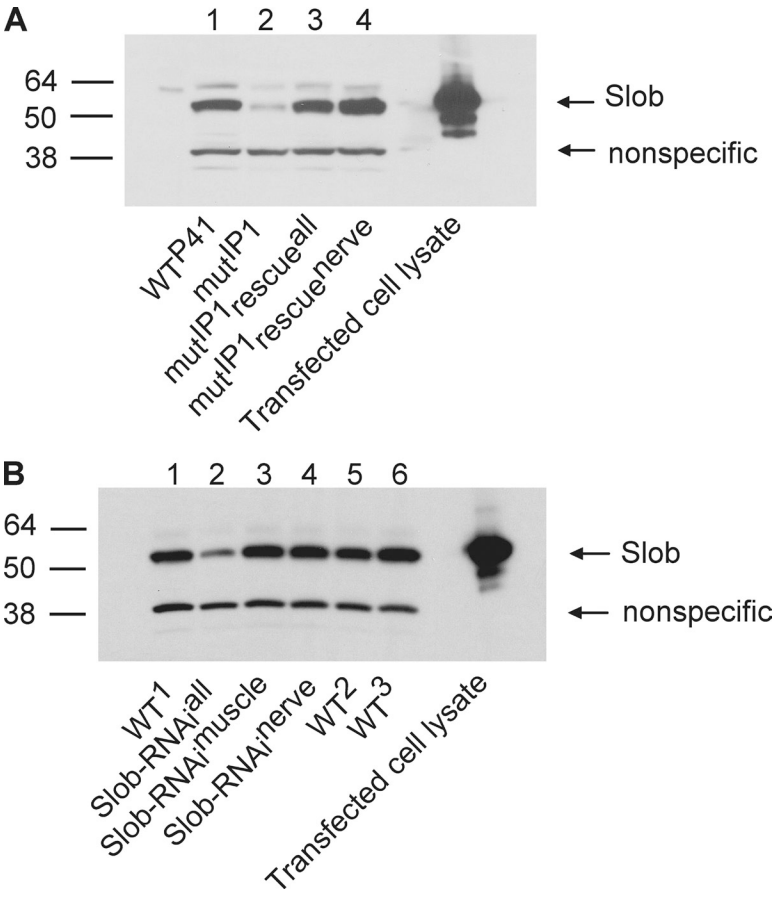

Figure 1. Slob-null and RNAi flies show reduced Slob protein expression in adult fly heads. (A) In mut^{IP1} flies (lane 2), there is greatly reduced Slob expression compared with the WT^{P41} line (lane 1). The remaining staining near 57 kD in lane 2 is a result of the presence of a cross-reacting band not related to Slob. Slob expression driven by a ubiquitous (lane 3) or nervous system–specific elav-GS (lane 4) Gal4 driver in the mut^{IP1} background is able to restore Slob expression in the fly head. (B) In Slob-RNAi flies, expression of Slob-RNAi driven by the ubiquitous Gal4 driver reduces the expression of Slob protein (lane 2). In contrast, Slob-RNAi expression driven by either the muscle-specific Gal4 driver (lane 3) or the nerve-specific Gal4 driver 1407-Gal4 (lane 4) does not affect Slob levels in the adult head. WT^1 and WT^2 are the uncrossed parental lines used as WT controls (refer to Materials and methods); WT^3 is a yellow-white fly line used as an additional control.

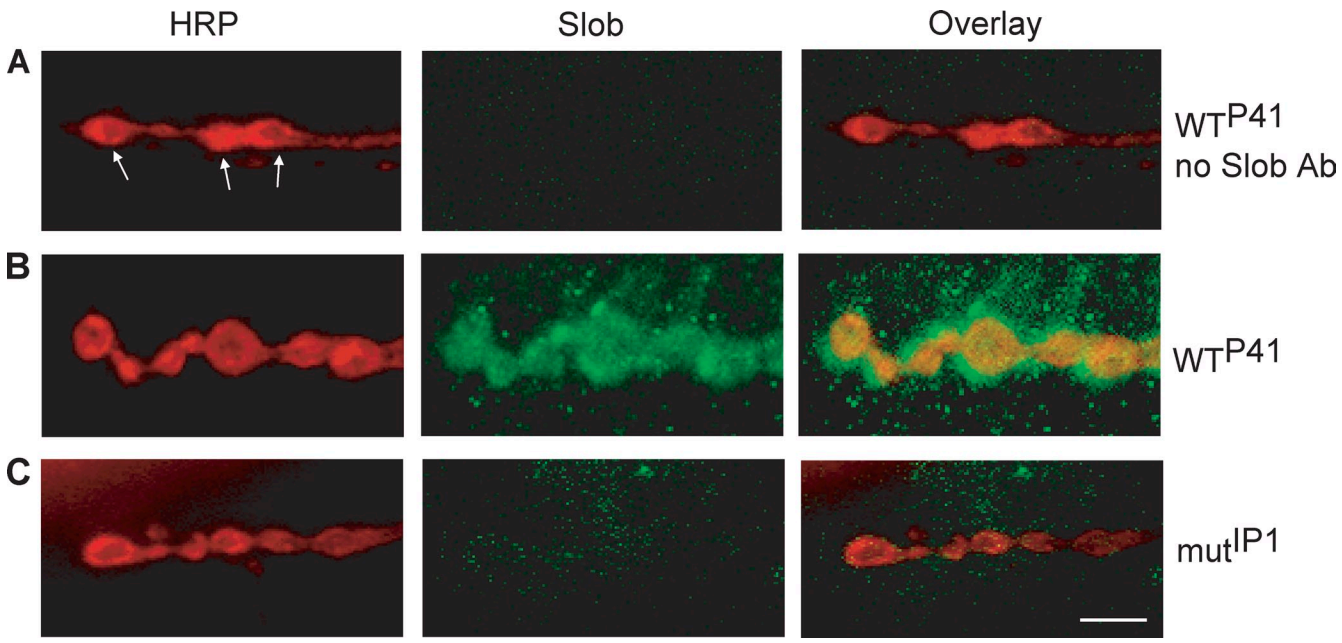

Figure 2. Slob expression at the *Drosophila* NMJ. HRP antibody stains nerve surface and presynaptic terminals (red; left column). White arrows in A point to individual synaptic boutons. Polyclonal antibody to Slob (green; middle column) stains Slob in nerve and nerve terminals and to a more limited extent in muscle. Overlay (yellow-orange; right column) illustrates colocalized Slob and HRP staining. (A) Staining of the WT line in the absence of primary antibody to Slob. (B) Staining of the WT line. (C) Staining of the Slob-null line. Bar, 5 μm.

we used an anti-HRP antibody to visualize nerve surface and presynaptic terminals (left columns in Figs. 2–4) and a Slob antibody to visualize Slob (middle columns in Figs. 2–4). The use of antibody against HRP to specifically identify nerve surface and terminals in *Drosophila* is well established (Jan and Jan, 1982; Sun and

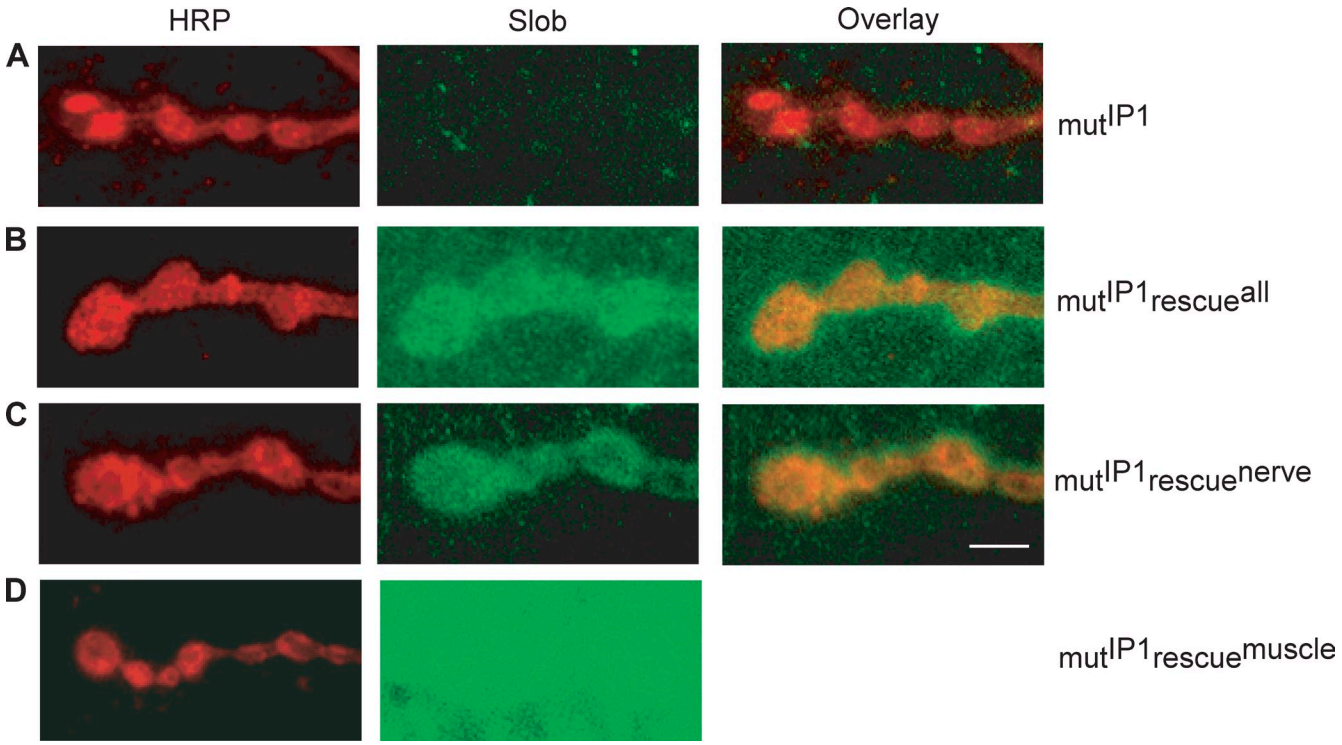

Figure 3. Slob can be restored to specific locations in a Slob-null background. Staining as for Fig. 2. (A) The Slob-null line as control. (B) Ubiquitous rescue of Slob. (C) Rescue of Slob expression in nerve. (D) Rescue of Slob expression in muscle. Bar, 5 μm.

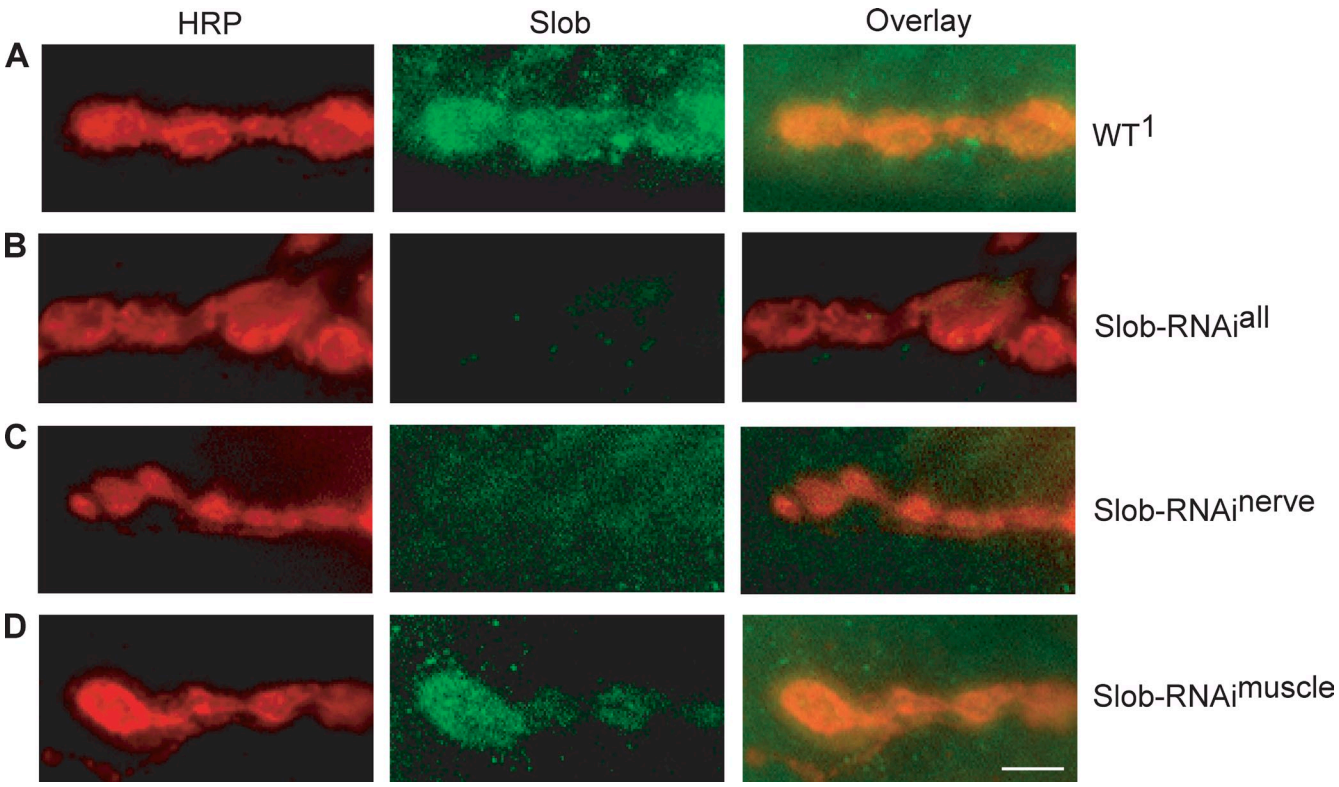

Figure 4. Slob can be disrupted in a tissue-specific manner using Slob-RNAi and specific Gal-4 drivers. Staining as for Figs. 2 and 3. (A) The uncrossed WT^{1} line (refer to Materials and methods) as control. (B) Ubiquitous disruption of Slob. (C) Disruption of Slob expression in nerve. (D) Disruption of Slob expression in muscle. Bar, 5 µm.

Salvaterra, 1995; Parrish et al., 2009; Paschinger et al., 2009; Shen and Ganetzky, 2009). Staining of WT^{P41} flies in the absence of the primary antibody against Slob shows no staining in boutons and a minimal amount of background (Fig. 2 A, middle). Slob is expressed in nerve and to a more limited extent in muscle in WT^{P41}

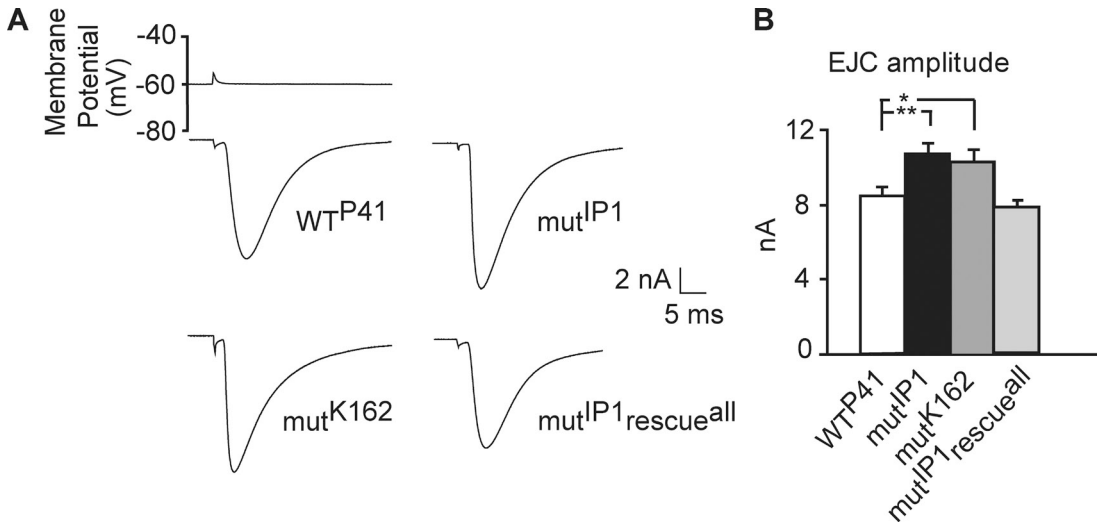

Figure 5. Evoked synaptic transmission is increased in Slob-null flies. (A) Representative recording of the muscle cell membrane potential during voltage clamping and sample traces. The EJC was measured with the muscle cell voltage clamped at −60 mV (top). The stimulation artifact arises from stimulation of the segmental nerve. Sample EJC traces from WT control, two Slob-null lines, and one ubiquitous rescue fly line are shown. (B) Pooled data. Peak amplitude of EJC is increased significantly in mut^{IP1} and mut^{K162} flies (black and dark gray bars) compared with the WT^{P41} control (white bar). In addition, ubiquitous expression of Slob57 in a Slob-null background rescues synaptic transmission to the WT level (light gray bar).

flies (Fig. 2 B). As shown in the merged image in Fig. 2 B (overlay, right panel), much but not all of the Slob expression overlaps with the HRP staining. In mut^{IP1} flies, in contrast, most or all of the Slob staining in both nerve and muscle is abolished (Fig. 2 C).

In the mut^{IP1} background (Fig. 3 A), ubiquitous rescue of Slob restores its expression in both nerve and muscle (Fig. 3 B). Rescue in the nervous system restores the overlapping Slob and HRP staining (Fig. 3 C), whereas rescue with the muscle-specific driver leads to high levels of Slob immunostaining in the muscle (Fig. 3 D).The extensive muscle staining in the $mut^{IP1}rescue^{muscle}$ line makes the overlay uninformative; therefore, it is not shown.

Similarly, ubiquitous knockdown of Slob ($Slob\text{-}RNAi^{all}$) decreases Slob expression in both nerve and muscle, and eliminates the overlap with HRP staining seen in WT^{1} flies (compare Fig. 4, A with B). Driving Slob-RNAi expression in nerve decreases Slob expression in the larval nerve terminals but not in muscle (Fig. 4 C), whereas Slob-RNAi expression in muscle leaves the nerve terminal expression intact (Fig. 4 D).

Slob knockout/knockdown leads to altered synaptic transmission

After confirming the changes in Slob expression in Slob-null and tissue-specific rescue flies, and the targeted

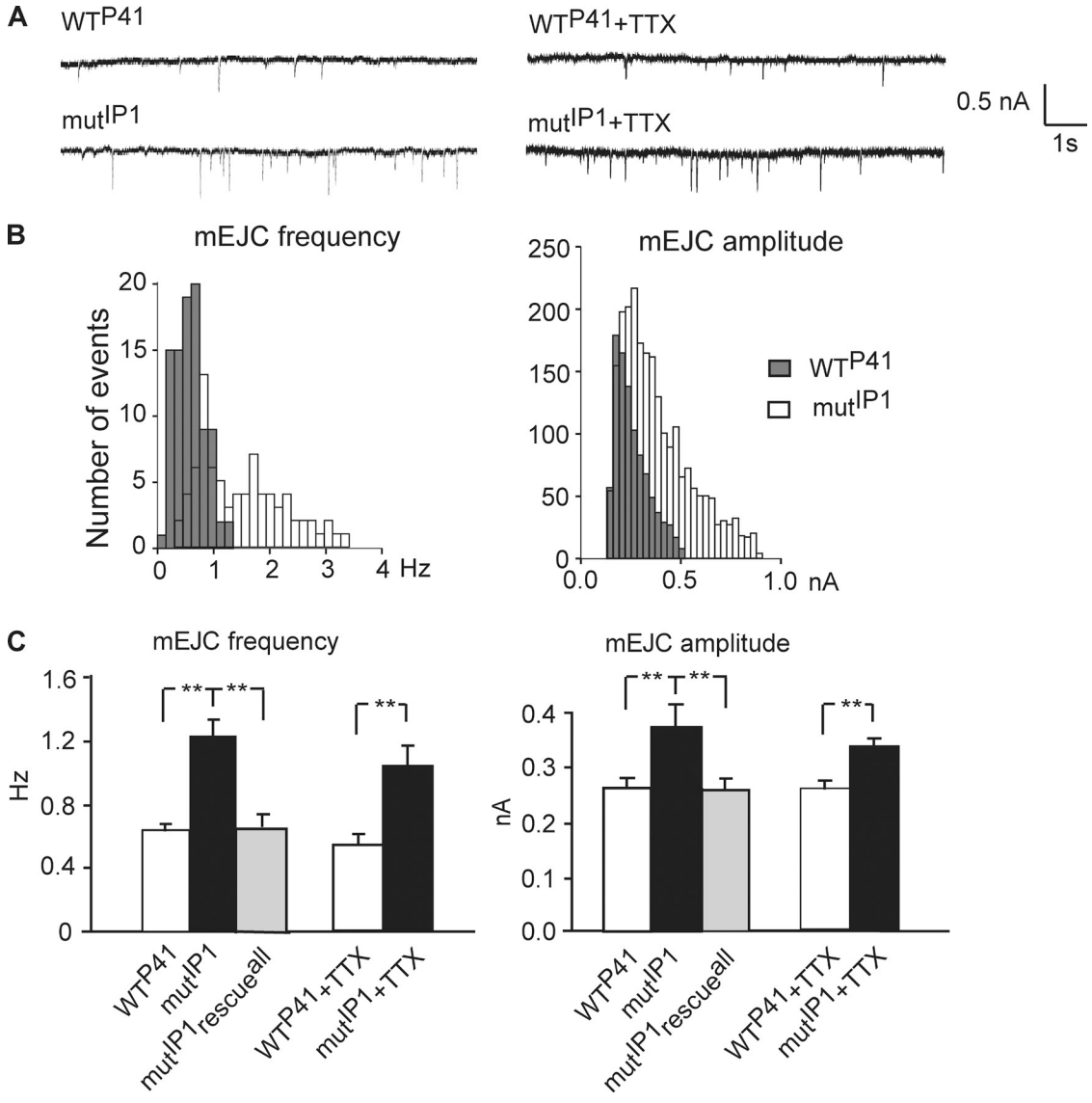

Figure 6. mEJC frequency and amplitude are increased in Slob-null flies. The muscle cell was voltage clamped at −60 mV as for Fig. 5, but no stimulus was delivered to the segmental nerve. (A) Sample mEJC traces from control and Slob-null flies in the absence or presence of 1 μM TTX. (B) mEJC frequency and amplitude distributions in WT^{P41} and mut^{IP1} flies. Bin sizes are 0.15 Hz and 0.05 nA, respectively. The distributions for mut^{IP1} are shifted dramatically to higher frequency and amplitude. (C) Pooled data. Frequency and amplitude of mEJCs are increased significantly in mut^{IP1} line (black bar) compared with WT^{P41} flies (white bar) in the absence or presence of TTX. In $mut^{IP1}rescue^{all}$ flies (gray bar), both mEJC frequency and amplitude are rescued to the level of the WT^{P41} flies.

TABLE I

EJC rise and decay times in mut^{IP1} and WT^{P41} flies

Fly genotype	Rise time	Decay time
	ms	*ms*
WT^{P41}	5.30 ± 0.23 (24)	50.85 ± 3.02 (24)
mut^{IP1}	6.17 ± 0.25 (21)	57.16 ± 5.36 (21)

Comparison of rise time and decay time reveals no difference between WT^{P41} and mut^{IP1} lines. Mean ± SEM is shown for each group. Rise time (ms) is defined as the time for the trace to rise from 10% of the peak amplitude to 90% of the peak amplitude. Decay time (ms) is defined as the time for the trace to decay from 90% of the peak amplitude to 10% of the peak amplitude. Cell numbers are shown in parentheses. For sample traces, see Fig. 5.

disruption of Slob expression in Slob-RNAi flies, we went on to determine whether Slob influences synaptic function at the *Drosophila* NMJ. We first measured the evoked EJC by clamping the muscle cell at −60 mV and stimulating the presynaptic nerve at 0.2 Hz, with an extracellular $CaCl_2$ concentration of 0.3 mM. We found that the EJC peak amplitude (sample traces shown in Fig. 5 A) in mut^{IP1} flies is ∼25% higher than in WT^{P41} flies ($P < 0.01$; Fig. 5 B). mut^{K162}, the other Slob-null fly line tested, also shows a significant increase in EJC peak amplitude compared with WT^{P41} ($P < 0.05$; Fig. 5, A and B). We also systematically examined the time course of the EJC and found no significant differences between WT and Slob-null flies (Table I). Finally, to confirm that it is the disruption of Slob, but not any other proteins, that induces the increase in EJC amplitude, we used the $mut^{IP1}rescue^{all}$ fly line that expresses Slob ubiquitously in the Slob-null background. We found that ubiquitous restoration of Slob is able to rescue the EJC peak amplitude to the WT level (Fig. 5, A and B).

Next, we asked whether manipulation of Slob expression leads to changes in spontaneous neurotransmitter release. We examined the frequency and amplitude of miniature EJCs (mEJCs) by clamping the muscle cells at −60 mV in the absence or presence of 1 μM TTX, without stimulating the innervating segmental nerve (extracellular $CaCl_2$ concentration, 0.3 mM). As shown in the sample traces in Fig. 6 A and pooled data in Fig. 6 C, TTX does not change the mEJC amplitude or frequency in mut^{IP1} or WT^{P41} flies, nor does it alter the marked differences in mEJCs between the mut^{IP1} and WT^{P41} lines. Accordingly, we performed other mEJC recordings in

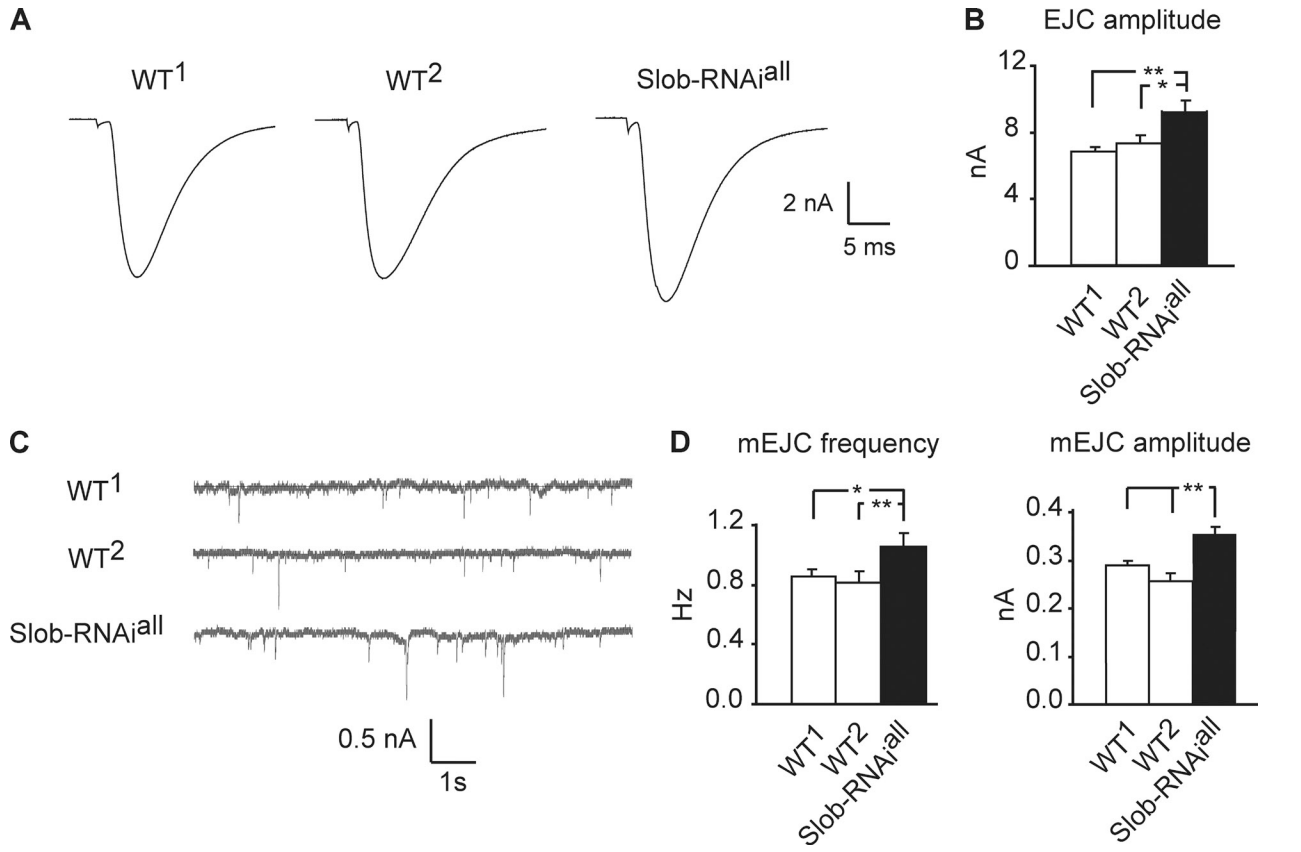

Figure 7. Evoked and spontaneous synaptic transmission are increased in Slob-RNAi flies. (A) Sample EJC traces from two control lines and one Slob-RNAi line. (B) Pooled data. Peak amplitude of EJC is increased significantly when Slob-RNAi is expressed ubiquitously (black bar) compared with controls (white bars). (C) Sample mEJC traces from two control lines and one Slob-RNAi line. (D) Pooled data. Frequency and amplitude of mEJCs are increased significantly in the $Slob\text{-}RNAi^{all}$ line (black bars) compared with controls (white bars). Refer to Materials and methods for the definition of the WT^1 and WT^2 lines.

the absence of TTX. The cumulative distributions of mEJC frequency and amplitude are shifted strongly to the right in mut^{IP1} as compared with WT^{P41} flies (Fig. 6 B). The pooled data in Fig. 6 C demonstrate that the mut^{IP1} fly line shows a remarkable increase in mEJC frequency, almost 100% higher than in WT^{P41} flies (in the absence or presence of TTX; $P < 0.01$ for both). The mEJC amplitude in the mut^{IP1} fly line is also significantly greater than in WT^{P41} flies (in the absence or presence of TTX; $P < 0.01$ for both). The mut^{K162} line also exhibits an enhancement in mEJC frequency and amplitude (unpublished data). As is the case for evoked synaptic transmission, ubiquitous restoration of Slob is able to rescue the mEJC peak amplitude and frequency to the WT levels (Fig. 6 C).

To further exclude the possibility that the altered synaptic transmission in these Slob-null flies is a result of the effects of the P-element other than disrupting the Slob gene, we studied synaptic transmission in Slob-RNAi flies. Sample EJC traces from Slob-RNAi flies are shown in Fig. 7 A. The two un-recombined parental lines (WT^1) and (WT^2) were used as controls. We found that flies with ubiquitous expression of Slob-RNAi (Slob-$RNAi^{all}$) exhibit an ~30% increase in EJC peak amplitude compared with the control lines ($P < 0.05$ and $P < 0.01$; Fig. 7 B). This similar enhancement of EJC peak amplitude in Slob-null and Slob-RNAi flies is consistent with the idea that the synaptic modulation results from the absence of Slob.

To determine whether the changes in mEJC frequency and amplitude are caused by the disruption of other genes by the P-element, again we examined Slob-RNAi flies. As shown in the sample traces (Fig. 7 C) and pooled data (Fig. 7 D), ubiquitous disruption of Slob (Slob-$RNAi^{all}$) produces significantly enhanced mEJC frequency and amplitude. Collectively, these data suggest strongly that it is the disruption of Slob expression that leads to the elevated spontaneous neurotransmitter release.

Slob modulates synaptic transmission via the dSlo potassium channel

We went on to investigate whether the altered synaptic transmission induced by the absence of Slob is mediated through changes in the dSlo channel. We first studied synaptic transmission in Slo4 flies in which the dSlo channel is disrupted (Atkinson et al., 1991). As shown in Fig. 8 B and Table II, there is no statistically significant difference in EJC amplitude between Slob WT and mutant flies in the Slo4 background ($P > 0.25$). There may be a modest change in the EJC decay kinetics, but this was not pursued further. Furthermore, mEJC frequency

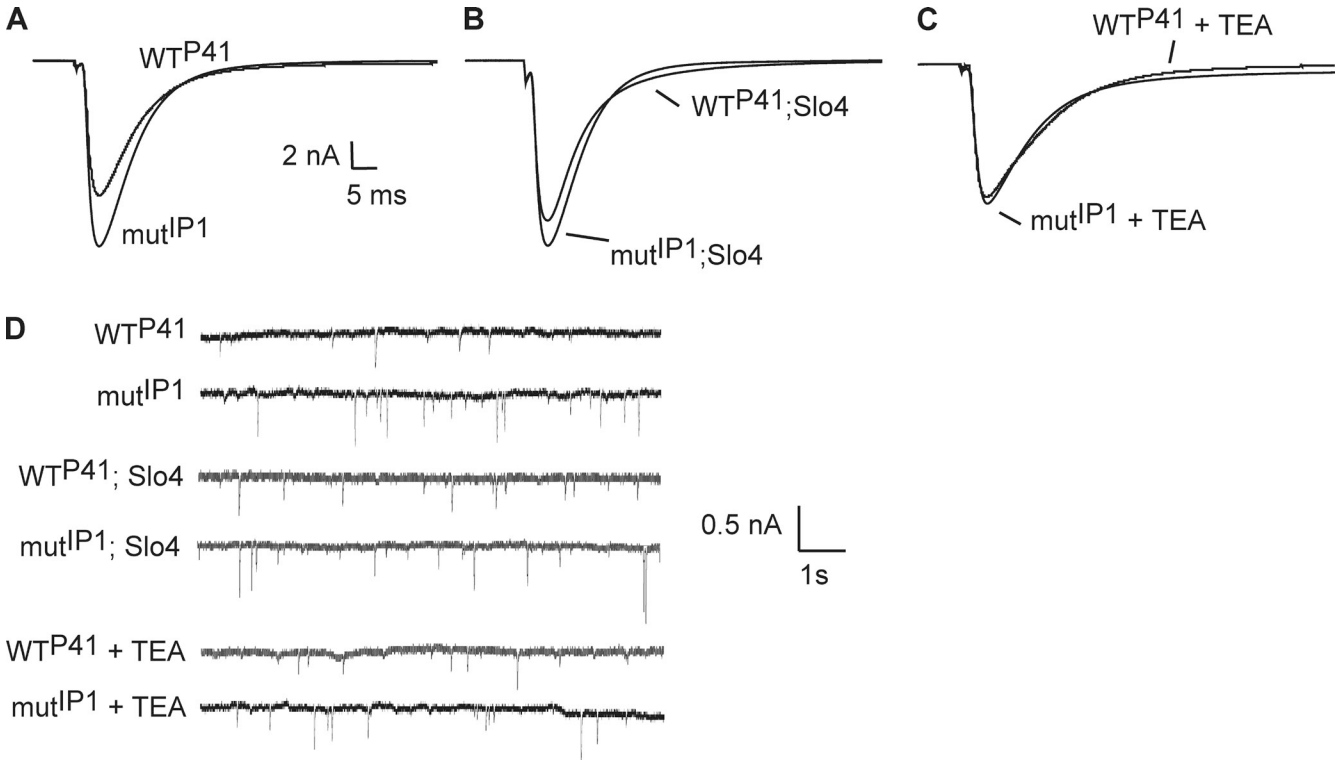

Figure 8. Genetic or pharmacological disruption of dSlo eliminates the differences in synaptic transmission between mut^{IP1} and WT^{P41} flies. (A) Averaged EJC traces from Slob-null and control lines. The difference in the EJC (A) is not observed in the Slo4 genetic background (B) or in the presence of 1 mM TEA (C). (D) Sample mEJC traces from Slo4 flies crossed to either Slob WT or null flies, or from control and Slob-null lines in the absence or presence of 1 mM TEA. Pooled data are shown in Table II.

TABLE II

dSlo mediates the difference in synaptic transmission between mut^{IP1} and WT^{P41} flies

Fly genotype/treatment	EJC amplitude	mEJC frequency	mEJC amplitude
	nA	*Hz*	*nA*
WT^{P41}	11.17 ± 0.73 (37)	0.94 ± 0.09 (48)	0.26 ± 0.02 (34)
mut^{IP1}	14.69 ± 0.62 (45)**	1.59 ± 0.17 (33)**	0.35 ± 0.02 (40)*
WT^{P41};Slo4	11.59 ± 0.94 (10)	1.45 ± 0.22 (13)	0.28 ± 0.02 (13)
mut^{IP1};Slo4	13.29 ± 1.11 (15)	1.68 ± 0.29 (10)	0.34 ± 0.02 (10)
WT^{P41}+TEA	10.39 ± 0.84 (14)	1.17 ± 0.19 (14)	0.24 ± 0.02 (10)
mut^{IP1}+TEA	11.72 ± 0.48 (38)	1.55 ± 0.17 (32)	0.28 ± 0.01 (34)

Slo4 genetic background, or TEA application, abolish the effects of Slob on EJC amplitude and on the frequency and amplitude of the mEJC. As indicated by the asterisks, the mut^{IP1} group is significantly different from the WT^{P41} group, with respect to all parameters measured. The inhibition of dSlo either genetically (Slo4 flies) or pharmacologically (1 mM TEA) eliminates the differences and makes Slob knockout and WT flies statistically indistinguishable. Mean ± SEM is shown for each group. Cell numbers are in parentheses. For averaged EJC traces and sample mEJC traces, see Fig. 8.

and amplitude are not affected by the Slob genotype in Slo4 flies (Fig. 8 D and Table II).

To examine further the role of dSlo in the synaptic actions of Slob, we applied 1 mM of the pharmacological reagent TEA, which blocks recombinant dSlo channels with a K_i of 80 μM (Shen et al., 1994). Other cloned *Drosophila* potassium channels such as Shaker, Shab, Shaw, and Shal lack a critical tyrosine residue near their selectivity filters that is present in dSlo, and as a result, they are much less sensitive than dSlo to extracellular TEA

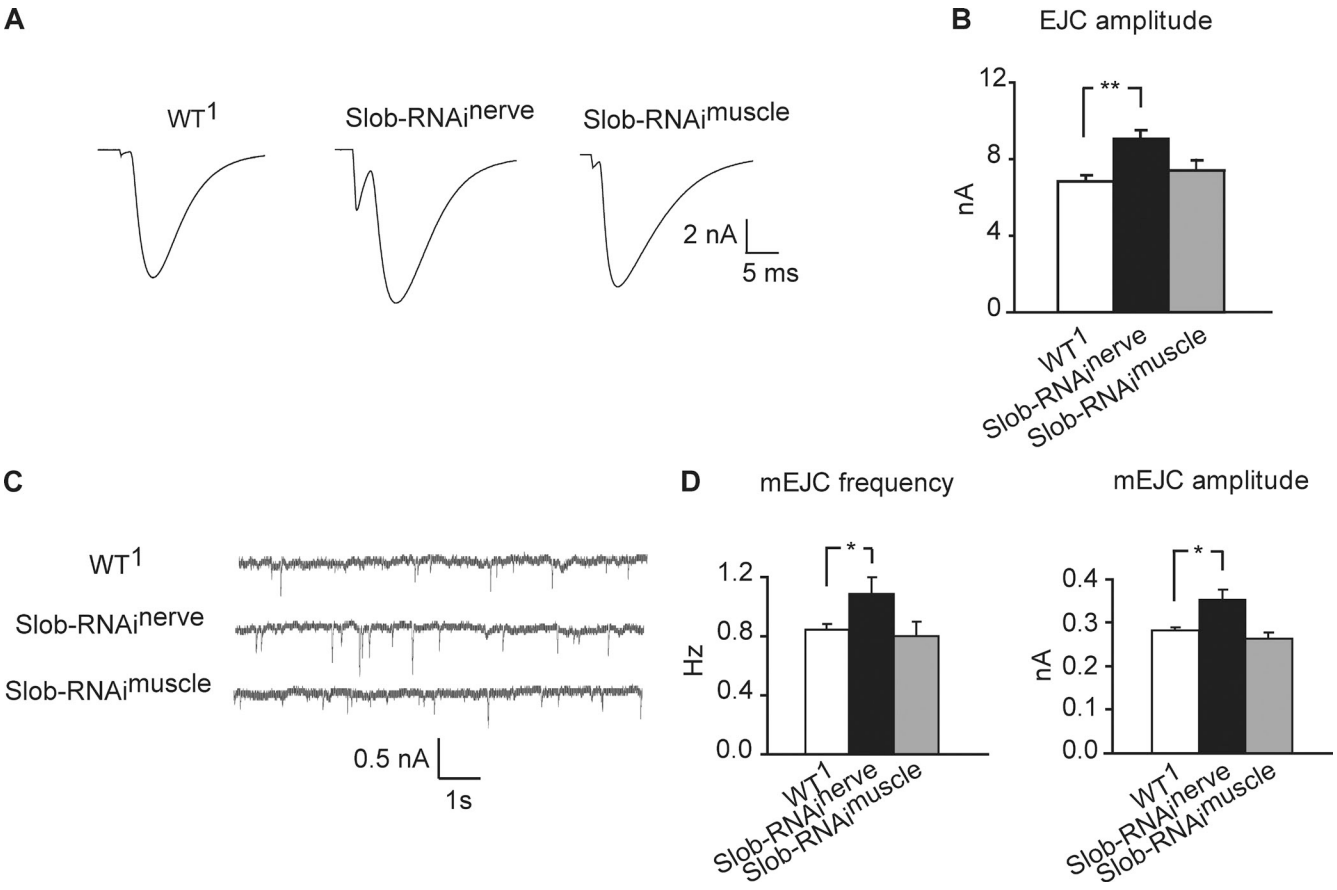

Figure 9. Targeted nerve expression of Slob-RNAi enhances evoked and spontaneous synaptic transmission. (A) Sample EJC traces from one control line and two Slob-RNAi lines. The Slob-RNAi^{nerve} line expresses Slob-RNAi in nerves, whereas the Slob-RNAi^{muscle} line expresses Slob-RNAi in muscle. (B) Pooled data. Peak amplitude of EJC is increased significantly when Slob-RNAi is expressed in nerves (black bar) compared with the uncrossed WT^1 control (white bar). EJC in the Slob-RNAi^{muscle} line (gray bar) is not significantly different from the EJC in the WT^1 control. (C) Sample mEJC traces from one control line and two Slob-RNAi lines. (D) Pooled data. Frequency and amplitude of mEJCs are increased significantly in the Slob-RNAi^{nerve} line (black bar), but not in the Slob-RNAi^{muscle} line (gray bar), compared with the uncrossed WT^1 control (white bar). Refer to Materials and methods for the definition of the WT^1 line.

(Kavanaugh et al., 1991). We found that the difference in EJC amplitude between Slob-null and control flies (Fig. 8 A) is no longer observed in the presence of 1 mM TEA (Fig. 8 C and Table II). Furthermore, TEA abolishes the difference in mEJC frequency and amplitude between WT and Slob-null flies (Fig. 8 D and Table II). Collectively, these complementary pharmacological and genetic results suggest an essential role for the dSlo channel in the effects of Slob on synaptic transmission.

Presynaptic Slob is critical for the regulation of synaptic function

To determine whether presynaptic or postsynaptic Slob participates in the regulation of synaptic transmission, we constructed Slob-RNAi flies in which Slob is knocked down either in nerve or in muscle (Slob-RNAi^{nerve} and Slob-RNAi^{muscle}, respectively). Slob knockdown in the nerve, but not in the muscle, leads to significantly increased EJC peak amplitude (Fig. 9, A and B). Similarly, Slob knockdown in the nerve leads to enhancement in

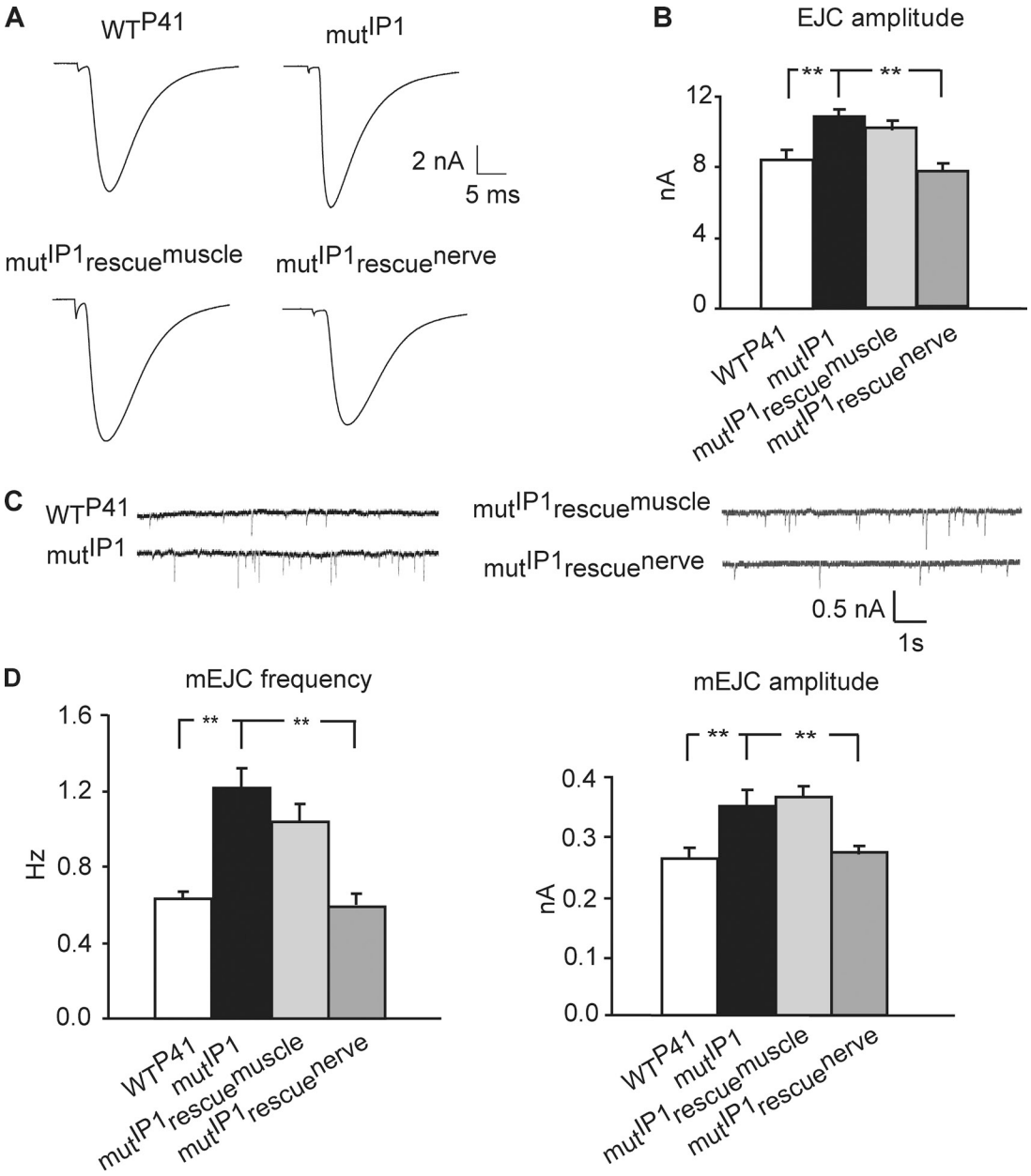

Figure 10. Rescue of Slob in nerves rescues the alterations in synaptic transmission. (A) Sample EJC traces from WT, Slob-null, and two rescue lines (rescue in muscle or nerve, respectively). (B) Pooled data. The enhanced EJC peak amplitude in Slob-null flies (black bar) is rescued by adding Slob back to the presynaptic nerve (dark gray bar), but not to the postsynaptic muscle (light gray bar). (C) Sample mEJC traces from WT, Slob-null, and two rescue lines (rescue in muscle or nerve, respectively). (D) Pooled data. Enhanced frequency and amplitude of non-evoked synaptic transmission (black bars) are rescued by adding Slob back to the presynaptic nerve (dark gray bars), but not to the postsynaptic muscle (light gray bars).

mEJC frequency and amplitude, whereas disruption of Slob in the muscle has no effect (Fig. 9, C and D). These data are consistent with the idea that presynaptic Slob regulates neurotransmitter release.

To further test the notion that presynaptic Slob participates in the regulation of synaptic transmission, we attempted to rescue the synaptic phenotype in the mut^{IP1} background by expressing Slob57 under the control of specific Gal4 drivers. As shown in Fig. 10 (A and B), restoring Slob to the segmental nerve (mut^{IP1}rescue^{nerve}) rescues the change in EJC amplitude, but restoring Slob to the postsynaptic muscles (mut^{IP1}rescue^{muscle}) fails to rescue. Similarly, the changes in mEJC amplitude and frequency (Fig. 10, C and D) in Slob-mutant flies can both be rescued to the level of the WT by adding Slob back to mut^{IP1} flies in the segmental nerve, but not in the muscle. These results indicate that presynaptic but not postsynaptic Slob participates in the regulation of synaptic transmission at the *Drosophila* NMJ.

Slob ablation alters synaptic transmission at various extracellular calcium concentrations but does not change muscle cell properties

Because dSlo is a calcium-dependent potassium channel and synaptic transmission at the *Drosophila* NMJ depends on calcium, we asked whether the regulation of synaptic transmission by Slob is calcium dependent. As shown in Fig. 11 A, Slob ablation alters the EJC amplitude over a range of calcium concentrations, although the effect of Slob appears to be less or absent at the highest calcium concentration tested (2 mM). Slob ablation elicits similar changes in mEJC amplitude (Fig. 11 B) and frequency (Fig. 11 C) at all the calcium concentrations we tested, indicating that the effect of Slob on spontaneous synaptic transmission is calcium independent. Finally, we asked if the enhanced synaptic transmission could be caused by changes in the size or other properties of the postsynaptic muscle cell. We analyzed the input resistance (Fig. 11 D) and capacitance (Fig. 11 E) of muscle cells and found no significant difference in either between the Slob-null and control flies.

DISCUSSION

In previous studies, we found that Slob is expressed at NMJ and in many brain areas, and that Slob modulates the voltage dependence of dSlo activation when the two proteins are expressed together in heterologous cells (Zhou et al., 1999; Jaramillo et al., 2004, 2006; Zeng et al., 2005). Furthermore, in vivo patch recordings

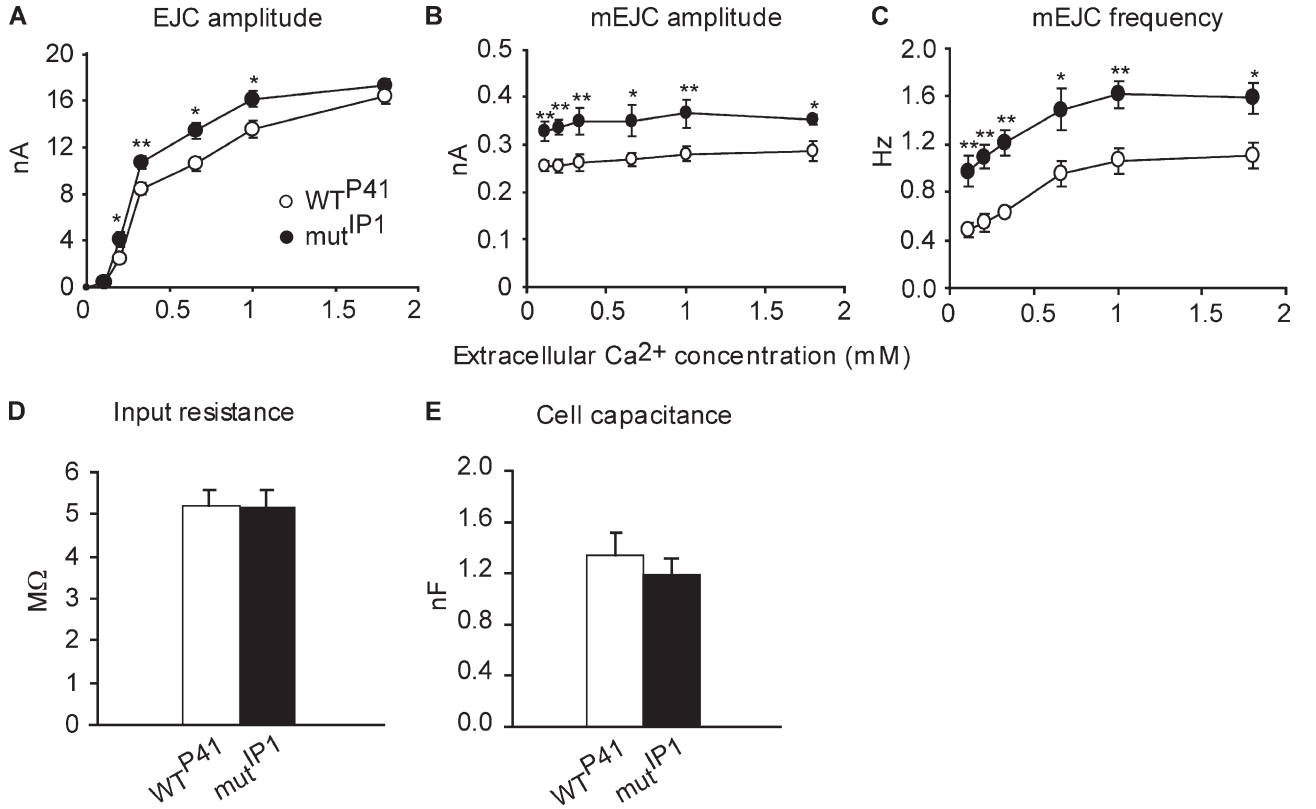

Figure 11. Calcium dependence of synaptic transmission, and postsynaptic cell properties, in Slob-null and WT flies. (A–C) Slob modulates various aspects of synaptic transmission: (A) EJC amplitude, (B) mEJC amplitude, and (C) mEJC frequency over a range of calcium concentrations. Input resistance (D) and cell capacitance (E) of the postsynaptic muscle cells are similar in WT^{P41} (white bar) and mut^{IP1} (black bar) lines.

from PI neurons in Slob-null and Slob-RNAi flies are consistent with the modulation of neuronal dSlo by Slob (Shahidullah et al., 2009). In the current study, we demonstrate that presynaptic Slob modulates both spontaneous and evoked synaptic transmission in vivo, via its regulation of the dSlo channel.

We find that knockout or knockdown of Slob increases the amplitude of evoked and spontaneous EJCs, and increases the frequency of spontaneous neurotransmitter release. The elevated mEJC and EJC amplitude could, theoretically, be caused by increased neurotransmitter release or the elevated response of postsynaptic glutamate receptors, or both. However, because the mEJC frequency reflects the rate of spontaneous transmitter vesicle exocytosis, our results suggest that in the absence of Slob, there is an increase in the probability of transmitter exocytosis from presynaptic boutons. This is supported by the observation that adding Slob back presynaptically rescues all aspects of the synaptic phenotype, and the additional finding that disrupting presynaptic Slob is necessary and sufficient to elicit the phenotype. In addition, when we add Slob back only to the postsynaptic muscle cells, the elevated spontaneous and evoked synaptic transmission cannot be rescued, suggesting that the actions of Slob in regulating NMJ synaptic transmission are exclusively presynaptic.

The dSlo channel is important for a variety of functions, including cell membrane repolarization. For example, in larval muscle cells lacking dSlo, action potential occurrence is facilitated (Singh and Wu, 1990). We used both genetic and pharmacological disruption of dSlo to examine its role in the synaptic actions of Slob. We find that synaptic transmission, measured in either the Slo4 genetic background or in the presence of a low concentration of TEA, is no longer affected by the manipulation of Slob expression. Collectively, these data imply that the effect of Slob on synaptic transmission is via its actions on dSlo. Although the selectivity of TEA for dSlo channels has not been tested in vivo, our conclusion that dSlo is important for the synaptic actions of Slob is supported strongly by the finding that both genetic and pharmacological inhibition of dSlo eliminate the effects of Slob mutation. Interestingly, we find that TEA predominantly alters EJC kinetics, whereas the Slo4 mutation that disrupts dSlo expression (Atkinson et al., 1991) primarily causes an increase in EJC amplitude. We have not investigated this apparent discrepancy, but it might reflect compensatory mechanisms in Slo4 flies that are not seen with acute channel block by TEA. Finally, we found (unpublished data) that Slob does not change the activity of the ether-a-go-go channel, to which Slob also binds (Schopperle et al., 1998). Thus, it seems likely that Slob regulates *Drosophila* NMJ function by modulating presynaptic dSlo channels.

Previously, we found that the predominant Slob57 isoform shifts the dSlo conductance–voltage relationship to more depolarized voltages and leads to channel inactivation and a faster deactivation of dSlo (Zeng et al., 2005). These data, collectively, suggest that there is likely elevated dSlo activity in Slob-null and RNAi flies. This is confirmed by our finding that the G-V relationship in PI neurons is shifted in the hyperpolarizing direction in Slob-null and RNAi flies (Shahidullah et al., 2009). How might elevated activity of neuronal dSlo lead to enhanced synaptic transmission? It is known that dSlo channel mutations cause a broadening of action potentials in *Drosophila* muscle cells (Elkins et al., 1986; Elkins and Ganetzky, 1988; Singh and Wu, 1990) and neurons (Saito and Wu, 1991). Inhibition of dSlo with TEA in WT flies increases the duration of the EJC (compare the durations of the WT^{P41} traces in Fig. 8, A and B), consistent with a broadening of the presynaptic action potential. Although it is generally believed that elongated action potentials contribute to enhanced transmitter release, this is not always the case. For example, the shortening of presynaptic action potentials leads to increased neurotransmitter release and a larger excitatory junctional potential at the jellyfish NMJ (Spencer et al., 1989). In addition, mutation of dSlo in *Drosophila* can lead to an apparently anomalous reduction in transmitter release, manifested as a reduced excitatory junctional potential and EJC (Warbington et al., 1996). Similarly, the mutation of dSlo and Shaker together significantly reduces the EJC slope at the *Drosophila* NMJ (Gho and Ganetzky, 1992). Although such findings may be counterintuitive, they are entirely consistent with ours, which demonstrate reduced synaptic transmission when Slob is present and dSlo activity is thereby decreased. It is conceivable that compensatory mechanisms, for example changes in the expression or trafficking of dSlo or other potassium channels, contribute to the apparently anomalous synaptic phenotype in Slob-null flies. In addition, the calcium influx that is necessary for neurotransmitter release will be influenced profoundly by such factors as calcium channel inactivation and by the driving force on calcium while the voltage-dependent calcium channels are open. Slob, by increasing action potential duration (Shahidullah et al., 2009), may increase calcium channel inactivation and anomalously decrease calcium influx, as has been seen previously (Spencer et al., 1989; Warbington et al., 1996). Interestingly, in spite of the fact that dSlo is a calcium-dependent channel, we find that the actions of Slob (via dSlo) are largely independent of the extracellular calcium concentration. A full understanding of the synaptic actions of Slob at the larval NMJ may require a detailed examination of calcium dynamics in the presynaptic nerve terminals.

Another protein that interacts with Slob, 14-3-3, influences many physiological functions in flies, including learning, Ca^{2+}-regulated exocytosis, and more (Morgan and Burgoyne, 1992; Skoulakis and Davis, 1996; Broadie

et al., 1997; Zhou et al., 1999). One of these functions, the regulation of the dSlo channel, depends on 14-3-3 binding to dSlo via Slob (Zhou et al., 1999). Thus, the absence of 14-3-3 in the dSlo regulatory complex may also contribute to the abnormal synaptic function that we observe in Slob-null and RNAi flies. It is evident that further in vivo experiments will be necessary to determine the precise role of the dSlo-Slob–14-3-3 regulatory protein complex in the modulation of synaptic transmission.

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