

# ROP, the *Drosophila* Sec1 homolog, interacts with syntaxin and regulates neurotransmitter release in a dosage-dependent manner

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**The Sec1 family of proteins is thought to function in both non-neuronal and neuronal secretion, although the precise role of this protein family has not been defined. Here, we study the function of ROP, the *Drosophila* Sec1 homolog, in neurotransmitter release. Electrophysiological analyses of transgenic lines over-expressing ROP and syntaxin, a presynaptic membrane protein, indicate that ROP interacts with syntaxin *in vivo*. Characterization of four point mutations in ROP shows that they fall into two phenotypic classes. Two mutations cause a dramatic reduction in both evoked and spontaneous neurotransmitter release. In contrast, the other two mutations reveal an increase in evoked neurotransmission. Our data further show that neurotransmission is highly sensitive to the levels of ROP function. Studies on heterozygote animals indicate that half the amount of wild-type ROP results in a dramatic decrease in evoked and spontaneous exocytosis. Taken together, these results suggest that ROP interacts with syntaxin *in vivo* and is a rate-limiting regulator of exocytosis that performs both positive and inhibitory functions in neurotransmission.**  
*Keywords:* neurotransmitter release/ROP/secretion/synaptic transmission/syntaxin

## Introduction

Recent studies on the mechanisms underlying neurotransmission indicate that synaptic vesicle exocytosis is a specialized form of regulated secretion (for review, see Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994; Südhof, 1995). Biochemical purification of synaptic vesicle proteins (Jahn and Südhof, 1994) and genetic analyses in yeast (Novick *et al.*, 1980) have identified a number of proteins that play important roles in secretion, providing a common molecular framework for neuronal and non-neuronal exocytosis. Synaptobrevin/VAMP, SNAP-25 and syntaxin are three such proteins thought to be key components of the release machinery. Synaptobrevin is an integral vesicle protein (Trimble *et al.*, 1988), while SNAP-25 and syntaxin are found in the plasma membrane

(Oyler *et al.*, 1989; Bennett *et al.*, 1992). Genetic analyses in yeast and fruit flies have demonstrated that these proteins play critical roles in exocytosis (Novick *et al.*, 1980; Aalto *et al.*, 1993; Protopopov *et al.*, 1993; Broadie *et al.*, 1995; Schulze *et al.*, 1995; Sweeney *et al.*, 1995). In addition, each of these three proteins is a specific target of clostridial neurotoxins which block neurotransmitter release, underscoring their importance in neuronal secretion (Schiavo *et al.*, 1992; Blasi *et al.*, 1993a,b). Together with the observation that syntaxin, SNAP-25 and synaptobrevin form a complex *in vitro* (Sollner *et al.*, 1993; Chapman *et al.*, 1994; Hayashi *et al.*, 1994; Pevsner *et al.*, 1994a), these data suggest that these proteins constitute a core complex mediating synaptic vesicle exocytosis.

Although syntaxin, synaptobrevin and SNAP-25 have been proposed to participate in the targeting and/or docking of synaptic vesicles to the presynaptic membrane (Bennett *et al.*, 1992; Sollner *et al.*, 1993), more recent data indicate that these proteins play a post-docking role. Syntaxin and SNAP-25 are not specific to the synapse, but are also localized throughout the axon (Garcia *et al.*, 1995; Sesack and Snyder, 1995; Schulze and Bellen, 1996), suggesting that these proteins alone do not provide specificity for targeting of synaptic vesicles. Furthermore, when toxins which cleave synaptobrevin are injected into squid nerve terminals, neurotransmitter release is inhibited, and there is an increase in the number of docked vesicles (Hunt *et al.*, 1994). Studies in *Drosophila* have shown that flies that lack syntaxin or n-synaptobrevin exhibit defects in evoked and spontaneous neurotransmission (Schulze *et al.*, 1995; Sweeney *et al.*, 1995) and an increase in the number of docked vesicles (Broadie *et al.*, 1995). These observations suggest that these proteins participate in a post-docking event, perhaps in the fusion process itself.

Although there is considerable evidence implicating the core complex as a key component of the exocytotic machinery, there is less known about proteins which may regulate neurotransmitter release. Since the plasticity of neurotransmitter release is believed to underly presynaptic aspects of learning and memory (Kandel, 1981; Hawkins *et al.*, 1993), the exocytotic process is likely to be subject to many different levels of regulation. One potential molecular target for such regulation is the core complex itself. Syntaxin, SNAP-25 and synaptobrevin spontaneously form an SDS-resistant complex *in vitro* (Hayashi *et al.*, 1994). Therefore, *in vivo*, there may be proteins that modulate this interaction or control the availability of individual components. For example, complexins have been suggested to directly regulate protein interactions with the core complex (McMahon *et al.*, 1995). An attractive candidate for a protein that may regulate exocytosis by modulating core complex function is the cytosolic *Drosophila* protein ROP, which is related to the yeast Sec1 protein.

Homologs of Sec1 have been identified in *Caenorhabditis elegans* (UNC-18), *Drosophila* (ROP) and rat (Munc-18/n-Sec1/rbSec1) (Hosono *et al.*, 1992; Hata *et al.*, 1993; Salzberg *et al.*, 1993; Garcia *et al.*, 1994; Pevsner *et al.*, 1994b). *sec1* mutants in yeast accumulate post-Golgi vesicles at the restrictive temperature, demonstrating a block at a late step in secretion (Novick *et al.*, 1980). *Caenorhabditis elegans unc-18* mutants show a paralytic phenotype (Brenner, 1974; Hosono *et al.*, 1992), and *rop* mutants display electroretinogram (ERG) defects consistent with altered synaptic transmission within the visual system (Harrison *et al.*, 1994), suggesting that Sec1-like proteins function in synaptic transmission. The functions of Sec1 family members may be mediated by their interaction with syntaxin family members. For example, overexpression of the yeast syntaxin homologs SSO1 or SSO2 can partially suppress a partial loss-of-function *sec1* mutation (Aalto *et al.*, 1993). *In vitro* studies have shown that the mammalian Sec1 homolog (Munc-18/n-Sec1/rbSec1), as well as ROP, tightly binds specific syntaxin isoforms (Hata *et al.*, 1993; Garcia *et al.*, 1994; Pevsner *et al.*, 1994b; Halachmi *et al.*, 1995). Although it seems clear that syntaxins and members of the Sec1 family can interact *in vitro*, it has been more difficult to address whether these proteins interact *in vivo*. For example, Garcia *et al.* (1995) were unable to detect a stable association between syntaxin and n-Sec1 using sucrose density gradient analysis and immunoprecipitation experiments, raising questions as to whether the syntaxin–n-Sec1 binding demonstrated *in vitro* is physiologically relevant.

In addition to a positive requirement for neuronal Sec1 family members in neurotransmitter release, an inhibitory role has also been proposed (Pevsner *et al.*, 1994a; Schulze *et al.*, 1994). n-Sec1 has been shown to inhibit SNAP-25 and synaptobrevin binding to syntaxin *in vitro*, therefore inhibiting formation of the core complex (Pevsner *et al.*, 1994a). We previously have demonstrated that overexpression of ROP causes a decrease in neurotransmitter release in a dose-dependent fashion, consistent with the hypothesis that ROP/Sec1 negatively regulates secretion, possibly by sequestering syntaxin (Schulze *et al.*, 1994).

To elucidate the role of ROP in neurotransmitter release, we have studied flies that overexpress syntaxin and ROP, and flies that carry partial loss-of-function mutations in *rop*. Our studies of ROP- and syntaxin-overexpressing lines show that syntaxin and ROP interact *in vivo*. Electrophysiological analysis of homozygous and heterozygous combinations of *rop* loss-of-function mutations yields three novel conclusions. First, ROP is required for a positive function in both evoked and spontaneous exocytosis. Second, ROP also performs an inhibitory function *in vivo*. Third, ROP is a rate-limiting factor in neurotransmitter release, suggesting that it is a key regulator of this process. Thus, we conclude that ROP acts as a rate-limiting regulator of exocytosis and performs both positive and inhibitory roles in secretion.

## Results

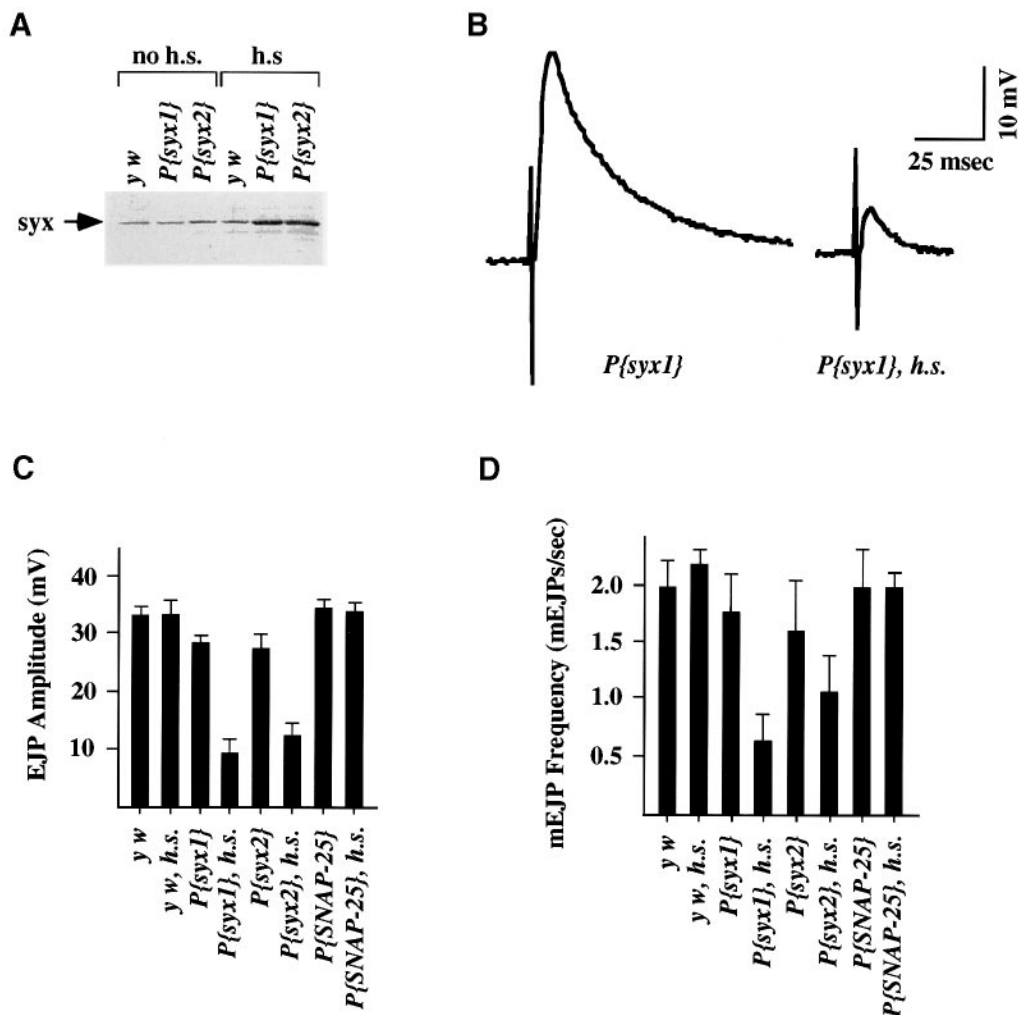
### **Overexpression of syntaxin decreases neurotransmitter release *in vivo***

To test whether a syntaxin–ROP complex functions in neurotransmitter release, we initiated electrophysiological

analyses of ROP- and syntaxin-overexpressing lines. We have shown previously that overexpression of ROP in *Drosophila* inhibits both evoked and spontaneous neurotransmitter release in a dose-dependent manner (Schulze *et al.*, 1994). To overexpress syntaxin *in vivo*, we generated two different transgenic lines (*P{syx1}* and *P{syx2}*) that carry the syntaxin cDNA under control of the hsp70 promoter (Pirrotta, 1988). *P{syx1}*, *P{syx2}* and control *y w* third instar larvae were heat shocked at 37°C for 1 h and allowed to recover for 1 h at room temperature. Larval brains were dissected and analyzed for syntaxin induction. Western analysis of larval brains demonstrates that this heat shock protocol induces syntaxin expression ~5-fold (Figure 1A). No induction is seen in *y w* controls after heat shock, or in uninduced *P{syx1}* and *P{syx2}* lines, when compared with *y w*. The additional syntaxin protein appears to localize properly, as heat-induced *P{syx1}* and *P{syx2}* larvae show a normal distribution of syntaxin in the neuropil of third instar larvae and at neuromuscular junctions (NMJs), and biochemical assays show it to be targeted normally to the membrane (data not shown).

To determine the effects of increased levels of syntaxin on neurotransmitter release, we measured the electrophysiological consequences at the third instar larval NMJ (Jan and Jan, 1976). Electrophysiological recordings were performed from third instar larval NMJs at muscle fiber 6 in abdominal segments 4–6. Overexpression of syntaxin in *P{syx1}* causes a 67% decrease in the amplitude of the excitatory junctional potential (EJP) ( $27.7 \pm 1.1$  mV in *P{syx1}* larvae versus  $9.2 \pm 1.9$  mV in heat-shocked *P{syx1}* larvae, unpaired Student's *t*-test,  $P < 0.0001$ ) (Figure 1B and C). In addition, the frequency of miniature EJPs (mEJPs) is decreased by 67% ( $1.8 \pm 0.3$  mEJPs/s in *P{syx1}* larvae versus  $0.6 \pm 0.2$  mEJPs/s in heat-shocked *P{syx1}*, unpaired Student's *t*-test,  $P < 0.01$ ), indicating that spontaneous release, in the absence of nerve stimulation, is also reduced (Figure 1D). Similar results were obtained for the *P{syx2}* line (Figure 1). The degree of the decrease of neurotransmitter release correlates with the amount of syntaxin overproduced (Spearman rank correlation,  $r = -0.90$ ,  $n = 5$ ). In contrast, *y w* control lines that were heat shocked, as well as non-heat shocked *P{syx1}* and *P{syx2}* larvae, showed no decrease in either EJP amplitude or mEJP frequency compared with *y w* controls.

In these transgenic lines, syntaxin is overproduced in all tissues upon heat shock. However, as has been shown for ROP overexpression (Schulze *et al.*, 1994), several lines of evidence suggest that overexpression of syntaxin does not have a general toxic effect on the muscles. First, visual inspection of muscles in heat-shocked *P{syx1}* and *P{syx2}* larvae showed no premature discoloration or vesiculation compared with wild-type. Second, the resting potentials for all heat-shocked muscles tested show no statistically significant differences from control muscles, suggesting no defects in the maintenance of muscle membrane potential (Table I). Third, mEJP amplitudes show no statistically significant differences from controls, suggesting that the observed defects are presynaptic in nature (Table I). Overexpression of syntaxin does not appear to impair recycling of synaptic vesicles, as tetanic stimulation did not reveal further decreases in neurotransmitter release (data not shown). These data suggest that syntaxin, when overexpressed, can act in a dominant-



**Fig. 1.** Overexpression of syntaxin causes a decrease in neurotransmitter release. **(A)** Immunoblots of brains and ventral nerve cords dissected from control *y w*, *P{syx1}* and *P{syx2}* larvae. Each lane contains proteins from five third instar larvae that were maintained at 24°C (no h.s.) or were given a 1 h heat shock at 37°C followed by a 1 h recovery at 24°C (h.s.). Proteins were resolved by SDS-PAGE. The syntaxin induction is ~5-fold in heat-shocked *P{syx1}* and *P{syx2}* lines when compared with non-heat-shocked lines and was quantitated using a Bio-Rad densitometer (analysis by Molecular Analysis). No induction is observed in controls after heat shock, or *P{syx1}* and *P{syx2}* before heat shock. Syntaxin migrates as a 35 kDa protein (arrow). **(B)** Amplitude of evoked responses in syntaxin-overexpressing third instar larvae. Representative excitatory junctional potentials (EJPs) at 1.5 mM  $Ca^{2+}$  are shown for *P{syx1}* in uninduced and induced conditions. The straight line before the response indicates the onset of nerve stimulation. The scale for the voltage trace is 10 mV, and the scale for the sweep speed is 25 ms. **(C)** Amplitude of evoked responses of *y w*, *P{syx1}*, *P{syx2}* and *P{SNAP-25}* larvae maintained at 24°C or treated with a 1 h heat shock. The amplitudes are plotted, and error is expressed as SEM. *P{syx1}*, h.s. larvae ( $n = 6$ ) exhibit a 67% decrease in EJP amplitude when compared with controls. *P{syx1}*, h.s. larvae ( $n = 6$ ) exhibit a 54% decrease compared with controls. No significant change is observed for *P{SNAP-25}*, h.s. larvae when compared with controls. **(D)** Frequency of spontaneous events in *y w*, *P{syx1}*, *P{syx2}* and *P{SNAP-25}* in uninduced and induced (h.s.) conditions. *P{syx1}*, h.s. larvae ( $n = 6$ ) display a 67% reduction in the frequency of mEJPs compared with controls. *P{syx2}*, h.s. larvae display a 32% reduction ( $n = 6$ ) in mEJP frequency when compared with controls. *P{SNAP-25}*, h.s. larvae do not significantly differ from controls. Error bars represent SEM.

negative manner, inhibiting specifically neurotransmitter release, perhaps by titrating out rate-limiting components of exocytosis.

To determine if defects in neurotransmission in syntaxin-overexpressing lines may be caused by non-specific effects of overexpression of a synaptic protein, we also examined the effects of ectopic expression of another member of the core complex, SNAP-25. The experimental paradigm used for *P{syx1}* and *P{syx2}* was repeated for two transgenic *P{SNAP-25}* lines. *P{SNAP-25}* larval brains were induced by heat shock and analyzed for induction by Western blotting. The induced SNAP-25 was overexpressed ~10-fold and was found in a crude membrane fraction (data not shown). In contrast to the overexpression of syntaxin, increased levels of SNAP-25 cause no defects

in either EJP amplitude or frequency of mEJPs (Figure 1C and D). Both EJP amplitude and mEJP frequency are similar in non-heat shock and heat shock conditions, as well as when compared with *y w* controls. Thus, overexpression of SNAP-25, a member of the core complex, does not decrease neurotransmitter release, suggesting that the inhibition of neurotransmitter release caused by syntaxin overexpression may be mediated through an interaction outside the core complex.

#### Syntaxin and ROP interact in vivo

Overproduction of either syntaxin or ROP alone strongly inhibits evoked and spontaneous neurotransmitter release. Simultaneous overexpression of both proteins might: (i) lead to an additive defect, further reducing neuro-

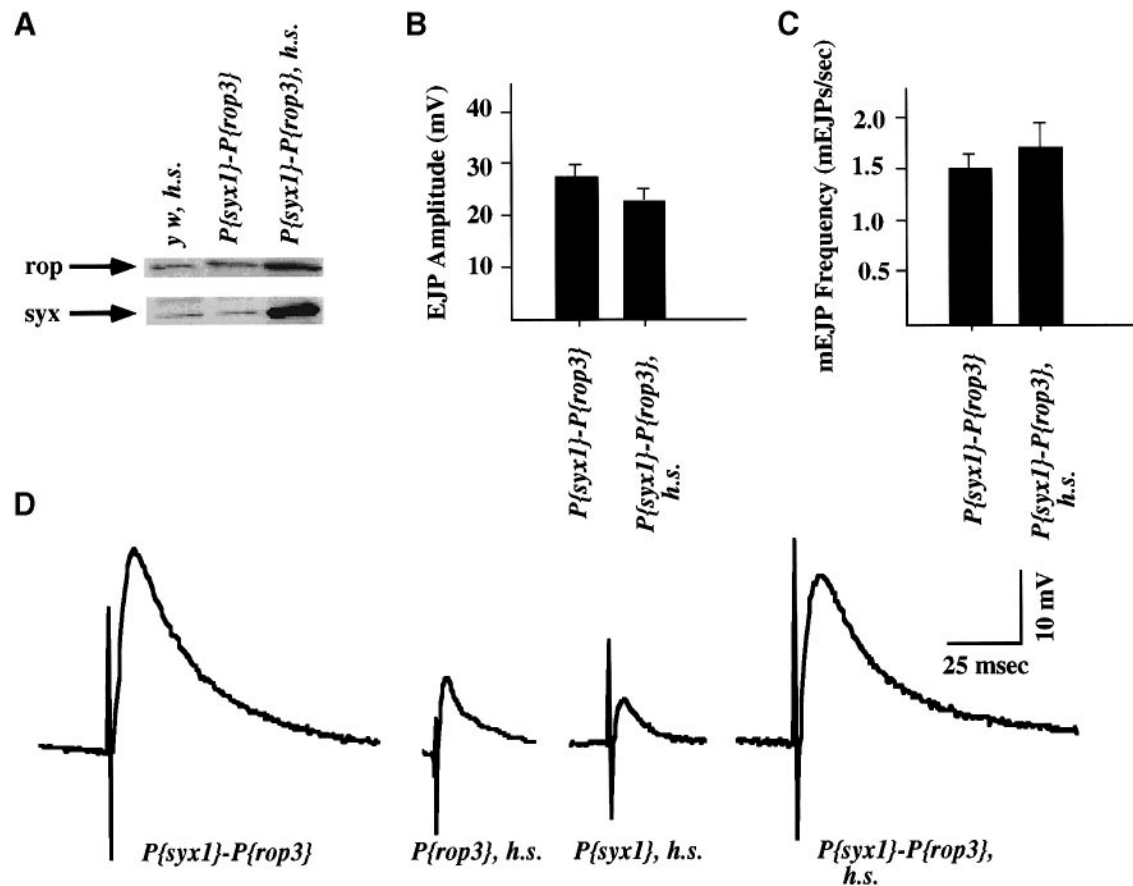
**Table I.** Electrophysiological properties of overexpression lines and *rop* mutations

Genotype	EJP amplitude (mV)	mEJP frequency (mEJP/s)	mEJP amplitude (mV)	Resting potential (mV)	<i>n</i>
1.5 mM Ca <sup>2+</sup>					
<i>y w</i>	33.1 ± 1.0	2.0 ± 0.2	0.78 ± 0.02	-55.8 ± 1.4	6
<i>y w, h.s.</i>	33.4 ± 2.1	2.2 ± 0.1	0.80 ± 0.01	-55.4 ± 1.4	6
<i>P{syx1}</i>	27.7 ± 1.1	1.8 ± 0.3	0.79 ± 0.02	-53.7 ± 1.2	7
<i>P{syx1}, h.s.</i>	9.2 ± 1.9	0.6 ± 0.2	0.75 ± 0.02	-51.8 ± 1.9	6
<i>P{syx2}</i>	26.7 ± 2.1	1.6 ± 0.4	0.77 ± 0.02	-54.5 ± 1.7	8
<i>P{syx2}, h.s.</i>	12.3 ± 1.5	1.1 ± 0.3	0.77 ± 0.01	-52.6 ± 0.7	6
<i>P{syx1}; P{rop3}</i>	27.2 ± 2.1	1.5 ± 0.1	0.79 ± 0.02	-57.4 ± 1.3	7
<i>P{syx1}; P{rop3}, h.s.</i>	23.3 ± 1.5	1.7 ± 0.2	0.82 ± 0.02	-58.0 ± 0.6	6
<i>P{SNAP-25}</i>	34.6 ± 1.2	2.0 ± 0.3	0.76 ± 0.02	-59.1 ± 0.7	6
<i>P{SNAP-25}, h.s.</i>	34.2 ± 1.2	2.0 ± 0.1	0.82 ± 0.04	-56.8 ± 1.1	6
<i>P{syx1}+</i>	29.0 ± 1.0	2.2 ± 0.3	0.77 ± 0.03	-57.3 ± 2.9	3
<i>P{syx1}+, h.s.</i>	20.3 ± 1.6	1.6 ± 0.2	0.70 ± 0.04	-56.0 ± 1.6	4
<i>P{syx2}+</i>	29.0 ± 0.9	2.1 ± 0.3	0.70 ± 0.04	-58.8 ± 1.6	4
<i>P{syx1}+, h.s.</i>	21.3 ± 1.9	1.6 ± 0.2	0.75 ± 0.06	-57.8 ± 1.7	4
<i>P{syx2}+; P{rop3}/+</i>	28.8 ± 3.3	2.1 ± 0.1	0.71 ± 0.04	-59.3 ± 2.6	4
<i>P{syx2}+; P{rop3}/+, h.s.</i>	25.0 ± 2.0	1.9 ± 0.3	0.73 ± 0.04	-54.3 ± 2.2	3
1 mM Ca <sup>2+</sup>					
<i>bw; st</i>	20.1 ± 1.6	3.3 ± 0.4	0.73 ± 0.01	-54.4 ± 1.5	20
<i>rop<sup>G11</sup>/rop<sup>G11</sup></i>	6.8 ± 2.1	1.2 ± 0.2	0.71 ± 0.04	-55.2 ± 1.6	9
<i>rop<sup>G11</sup>/rop<sup>A19</sup></i>	7.7 ± 2.1	0.9 ± 0.1	0.81 ± 0.05	-57.0 ± 2.2	6
<i>rop<sup>G11</sup>/+</i>	13.0 ± 2.3	1.5 ± 0.4	0.70 ± 0.05	-56.0 ± 1.5	5
<i>rop<sup>G27</sup>/+</i>	7.6 ± 0.4	0.8 ± 0.1	0.72 ± 0.06	-55.0 ± 2.1	5
<i>rop<sup>G11</sup>/rop<sup>F3</sup></i>	13.8 ± 2.1	1.9 ± 0.3	0.75 ± 0.04	-54.2 ± 1.0	8
<i>rop<sup>G11</sup>/rop<sup>G17</sup></i>	12.2 ± 2.3	1.3 ± 0.6	0.80 ± 0.05	-57.1 ± 1.5	6
<i>rop<sup>A19</sup>/rop<sup>F3</sup></i>	17.0 ± 2.3	1.6 ± 0.2	0.75 ± 0.06	-55.5 ± 2.2	4
<i>rop<sup>A19</sup>/rop<sup>G17</sup></i>	21.7 ± 4.9	2.2 ± 0.7	0.72 ± 0.04	-56.2 ± 2.7	4
<i>rop<sup>F3</sup>/+</i>	24.0 ± 1.3	2.7 ± 0.4	0.72 ± 0.04	-56.0 ± 2.5	5
<i>rop<sup>G17</sup>/+</i>	22.2 ± 1.8	2.8 ± 0.2	0.74 ± 0.03	-59.6 ± 3.9	5
<i>rop<sup>F3</sup>/rop<sup>F3</sup></i>	28.7 ± 0.9	3.0 ± 0.4	0.76 ± 0.03	-55.8 ± 1.7	6
<i>rop<sup>G17</sup>/rop<sup>G17</sup></i>	26.6 ± 2.3	2.6 ± 0.2	0.74 ± 0.03	-56.2 ± 1.1	8
<i>rop<sup>F3</sup>/rop<sup>G17</sup></i>	26.5 ± 1.8	3.9 ± 0.4	0.74 ± 0.05	-55.8 ± 1.75	5
0.6 mM Ca <sup>2+</sup>					
<i>bw; st</i>	2.9 ± 0.5	2.3 ± 0.2	0.75 ± 0.04	-57.3 ± 1.5	4
<i>rop<sup>G11</sup>/rop<sup>G11</sup></i>	1.7 ± 0.3	0.7 ± 0.1	0.75 ± 0.10	-55.0 ± 2.7	4
<i>rop<sup>A19</sup>/rop<sup>G11</sup></i>	2.5 ± 0.6	0.8 ± 0.2	0.74 ± 0.05	-58.3 ± 2.4	4
<i>rop<sup>G17</sup>/rop<sup>G17</sup></i>	10.5 ± 3.5	2.4 ± 0.1	0.73 ± 0.06	-56.3 ± 1.5	4
<i>rop<sup>F3</sup>/rop<sup>F3</sup></i>	9.2 ± 0.8	2.6 ± 0.5	0.78 ± 0.05	-58.5 ± 2.1	4
<i>rop<sup>F3</sup>/rop<sup>G17</sup></i>	15.3 ± 2.8	3.3 ± 0.3	0.71 ± 0.04	-58.3 ± 1.9	4
1.8 mM Ca <sup>2+</sup>					
<i>bw; st</i>	25.2 ± 2.0	2.0 ± 0.4	0.75 ± 0.05	-57.0 ± 1.0	6
<i>rop<sup>G11</sup>/rop<sup>G11</sup></i>	10.3 ± 1.3	1.0 ± 0.4	0.76 ± 0.04	-56.5 ± 2.4	4
<i>rop<sup>A19</sup>/rop<sup>G11</sup></i>	18.7 ± 2.7	1.0 ± 0.3	0.70 ± 0.03	-56.7 ± 1.7	8
<i>rop<sup>G17</sup>/rop<sup>G17</sup></i>	33.6 ± 3.7	2.0 ± 0.1	0.77 ± 0.05	-57.4 ± 2.2	5
<i>rop<sup>F3</sup>/rop<sup>F3</sup></i>	34.5 ± 1.4	3.0 ± 0.4	0.72 ± 0.04	-56.6 ± 1.7	6
<i>rop<sup>F3</sup>/rop<sup>G17</sup></i>	33.2 ± 4.3	3.0 ± 0.5	0.68 ± 0.05	-55.8 ± 2.8	4

Properties of the overexpression lines are shown, either without heat shock or with a 1 h heat shock at 37°C followed by a 1 h rest at 24°C (h.s.). Error is expressed as SEM. *n* = the number of independent larvae that were analyzed. Recordings for the overexpression lines were performed in HL-3 solution (Stewart *et al.*, 1994) in 1.5 mM Ca<sup>2+</sup>. Properties of *rop* mutant lines and heteroallelic combinations of *rop* alleles are shown. Recordings were performed in HL-3 solution with 0.6, 1.0 or 1.8 mM Ca<sup>2+</sup> as indicated.

transmitter release and suggesting that the inhibition by each protein is mediated through distinct pathways; (ii) show no significant difference from either ROP or syntaxin overexpression alone, suggesting that the inhibition by the two proteins converge into a single pathway; or (iii) restore neurotransmitter release to wild-type levels, indicating a direct interaction between the two proteins. We generated double transgenic lines that overexpress both syntaxin and ROP upon heat shock by crossing *P{syx1}* or *P{syx2}* with *P{rop3}*. Western analysis demonstrated that both syntaxin and ROP were overproduced upon heat shock (syntaxin ~7-fold and ROP ~4-fold

(Figure 2A). Interestingly, the simultaneous overexpression of both syntaxin and ROP suppresses the physiological defects seen when either is overexpressed alone. Evoked response was restored to ~85% of wild-type levels (27.2 ± 2.1 mV in *P{syx1}-P{rop3}*, 23.3 ± 1.5 mV in heat-shocked *P{syx1}-P{rop3}*) (Figure 2B and D). Although this suppression is very strong, it is not a complete suppression, which might be predicted if syntaxin and ROP function in a 1:1 complex, as has been suggested (Hata *et al.*, 1993; Pevsner *et al.*, 1994b). A possible reason for this is that the levels of overexpressed syntaxin and ROP are not equivalent. A similar suppression was



**Fig. 2.** Simultaneous overexpression of syntaxin and ROP suppresses the defects observed when either is overexpressed individually. (A) Immunoblots of brains and ventral nerve cords of *y w* and *P{syx1}-P{rop3}* larvae maintained either at 24°C or treated with a heat shock. Proteins from five third instar larvae were resolved by SDS-PAGE, and blots were probed for ROP and syntaxin. ROP migrates as a 66 kDa protein, while syntaxin migrates as a 35 kDa protein. ROP induction is ~3.5-fold and syntaxin induction is ~7-fold (analysis performed using NIH Image). (B) Amplitude of evoked responses of *P{syx1}-P{rop3}* larvae in uninduced and induced (h.s.) conditions. EJPs are plotted for *P{syx1}-P{rop3}* ( $n = 7$  independent larvae) and *P{syx1}-P{rop3}, h.s.* ( $n = 6$  independent larvae). Recordings were performed in 1.5 mM  $Ca^{2+}$ , and error is expressed as SEM. (C) Frequency of mEJPs in *P{syx1}-P{rop3}* larvae maintained at 24°C or treated with a 1 h heat shock at 37°C, followed by a 1 h recovery at 24°C (h.s.). The frequency of spontaneous events in *P{syx1}-P{rop3}, h.s.* ( $n = 6$  larvae) is restored to control levels (*P{syx1}-P{rop3}*) ( $n = 7$  larvae). Error is expressed as SEM. (D) Evoked responses of uninduced *P{syx1}-P{rop3}*, induced *P{rop3}*, induced *P{syx1}* and induced *P{syx1}-P{rop3}* larvae. Representative EJPs are shown. *P{rop3}, h.s.* larvae show a 45% reduction in EJP amplitude when compared with controls (see Figure 3 in Schulze *et al.*, 1994). *P{syx1}, h.s.* larvae show a 67% decrease in EJP amplitude (see Figure 1). Simultaneous overproduction of syntaxin and ROP suppresses the decreases observed when either is overexpressed alone. The straight line before the response indicates the onset of nerve stimulation.

observed for spontaneous responses (Figure 2C). Similar data were obtained for *P{syx2}-P{rop3}* transgenic animals (Table I). To address the specificity of this suppression, we examined double transgenics overexpressing both ROP and SNAP-25 upon heat shock and found that overexpressing SNAP-25 did not alter the inhibitory effects of overexpressing ROP alone (data not shown). Thus, the suppression of the electrophysiological phenotypes observed when syntaxin and ROP are overexpressed together is specific and indicates an *in vivo* interaction between ROP and syntaxin. We wished to determine if we could demonstrate this interaction *in vitro* by co-immunoprecipitation of a syntaxin-ROP complex from head extracts. Although the anti-syntaxin and anti-ROP antibodies that we used immunoprecipitated syntaxin and ROP respectively, we were not able to obtain evidence of co-immunoprecipitation of syntaxin and ROP. The difficulty of co-immunoprecipitating syntaxin and Sec1 homologs from tissue extracts has been documented by others (Garcia *et al.*, 1995) and underscores the importance of demonstrating an *in vivo* interaction.

It has been shown previously in *Drosophila* that altering the level of syntaxin or n-synaptobrevin at the synapse causes a decrease in neurotransmitter release and results in an increased number of docked vesicles (Broadie *et al.*, 1995; Schulze *et al.*, 1995; Sweeney *et al.*, 1995), suggesting that both of these members of the core complex function after vesicle docking. Overexpression of ROP also inhibits neurotransmitter release (see Figure 3 from Schulze *et al.*, 1994), which potentially could result from an inability of vesicles to dock. To address this possibility, we carried out ultrastructural analyses of the NMJs of *P{rop3}* and *P{rop3}* heat-shocked third instar larvae. Heat-shocked and non-heat-shocked *P{rop3}* larval synapses showed a similar amount of vesicles within a 15  $\mu$ m range from the membrane (heat shock,  $2.81 \pm 0.25$ ; no heat shock,  $2.21 \pm 0.37$ ;  $P = 0.062$ ). In addition, the number of docked vesicles was similar in *P{rop3}* and *P{rop3}* heat-shocked synapses ( $3.61 \pm 0.35$ ,  $3.21 \pm 0.39$ ,  $P = 0.503$ ). In addition, no obvious morphological defects were observed in *P{rop3}* heat-shocked larvae, suggesting that heat shock administration does not damage

	Df(3L) GN34	G27	A19	G11	G17	F3
Df(3L)GN34	-					
G27	-	-				
A19	-	-	-			
G11	+/-*	-	+	-		
G17	-	+/-*	+	+	+	
F3	-	-	+	+	+	+

-=no adult survivors  
 +=>20% expected adults  
 +/-<=5% expected adults

**Fig. 3.** *rop* mutations. Complementation analysis of *rop* mutations. *rop* mutations were tested for complementation with other *rop* alleles at room temperature. *rop<sup>G17</sup>* and *rop<sup>F3</sup>* alleles are both homozygous viable at 24°C, but semi-lethal at 28°C. *rop<sup>G27</sup>* behaves genetically as a null allele. *Df(3L)GN34* is a deficiency on the third chromosome that uncovers the *rop* locus. All mutations are lethal over the deficiency and a null allele, and the lethality of all *rop* mutations described here can be rescued by a genomic fragment spanning the *rop* gene (Harrison *et al.*, 1994), indicating that these mutations are partial loss-of-function mutations. The lethality of *rop<sup>F3</sup>* and *rop<sup>G17</sup>* observed at 28°C suggests that increased temperature may uncover additional defects in these lines.

the synapse. Note that heat shock of *P{rop3}* under similar conditions causes a 45% reduction in EJP amplitude (Schulze *et al.*, 1994). Therefore, although increasing the level of ROP protein inhibits neurotransmitter release, it does not impair the ability of synaptic vesicles to dock, suggesting that ROP may, like syntaxin and n-synaptobrevin, function after the docking of vesicles.

#### **ROP performs a positive or required function in evoked and spontaneous neurotransmission**

Mutations in the *rop* locus have been isolated previously (Harrison *et al.*, 1994). These include four point mutations which result in single amino acid substitutions [*rop<sup>A19</sup>* (H302→Y), *rop<sup>G11</sup>* (D45→N), *rop<sup>G17</sup>* (P254→S) and *rop<sup>F3</sup>* (R50→C)]. *rop<sup>G27</sup>* (Q311→Stop) behaves genetically as a null allele (Harrison *et al.*, 1994). Phenotypic characterization of the null allele indicates that ROP plays a role in general secretory events, as there are defects in the extracellular accumulation of normally secreted products (Harrison *et al.*, 1994). The four point mutations are lethal in *trans* to a deficiency that uncovers the region, as well as in *trans* to the null allele *rop<sup>G27</sup>* (Figure 3). In addition, all alleles can be rescued over a deficiency by a genomic construct spanning the *rop* locus. Therefore, these four mutations represent loss-of-function mutations (Harrison *et al.*, 1994). As shown in Figure 3, *rop<sup>A19</sup>* and *rop<sup>G11</sup>* are homozygous lethal, while *rop<sup>G17</sup>* and *rop<sup>F3</sup>* are homozygous viable at room temperature but are semi-lethal at 28°C. These point mutations do not noticeably affect total ROP protein levels as determined by Western analysis (data not shown).

To elucidate the role of ROP in neurotransmitter release, we studied the effects of the *rop<sup>A19</sup>*, *rop<sup>G11</sup>*, *rop<sup>G17</sup>* and *rop<sup>F3</sup>* mutations at the third instar larval NMJ. The physiological consequences of the complete absence of ROP function cannot be assessed at the NMJ because of gross morphological defects in *rop* null mutants (Harrison *et al.*, 1994). In addition, *rop<sup>A19/rop<sup>A19</sup></sup>* animals cannot be analyzed at the third instar larval NMJ, since they are embryonic lethal. However, *rop<sup>G11/rop<sup>G11</sup></sup>* animals are lethal at third instar larval/early pupal stages, and all

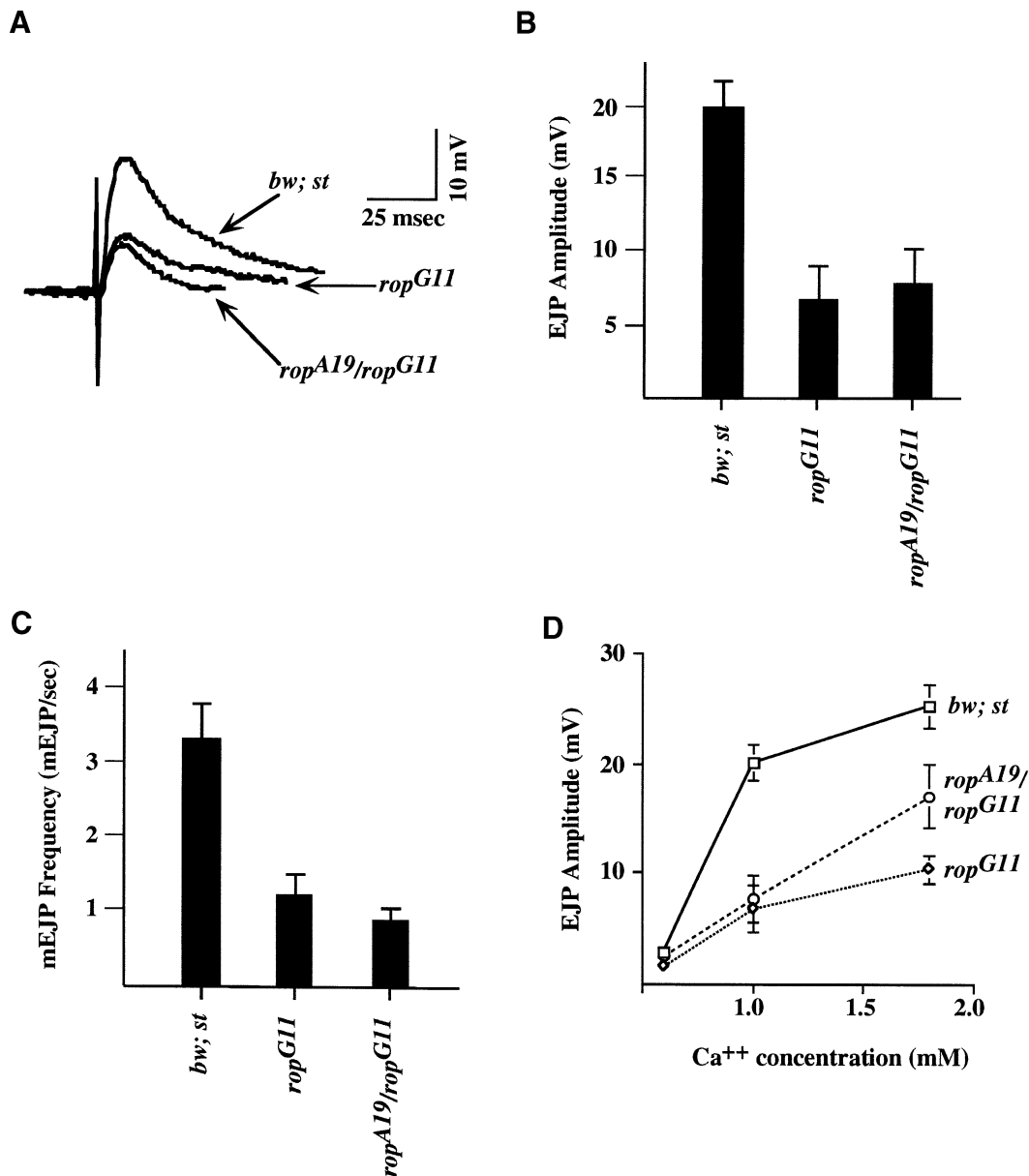
heteroallelic combinations of the four *rop* mutations are viable at room temperature, thereby allowing analysis of most *rop* mutant combinations. Interestingly, *rop<sup>A19/rop<sup>G11</sup></sup>* heterozygote animals are fully viable, whereas *rop<sup>A19</sup>* or *rop<sup>G11</sup>* homozygote animals are lethal. This finding, combined with the observation that the *rop<sup>A19</sup>* and *rop<sup>G11</sup>* alleles are also lethal in combination with a deficiency removing *rop*, suggests that these alleles exhibit intra-allelic complementation and are probably hypomorphic alleles.

At 24°C, *rop<sup>G11/rop<sup>G11</sup></sup>* third instar larvae could be obtained from non-crowded vials. These larvae were sluggish compared with control *bw; st* larvae, but showed no obvious morphological defects. As shown in Figure 4A and B, *rop<sup>G11/rop<sup>G11</sup></sup>* homozygotes displayed a 66% reduction in EJP amplitude when compared with *bw; st* controls ( $6.8 \pm 2.1$  mV in *rop<sup>G11/rop<sup>G11</sup></sup>* compared with  $20.1 \pm 1.6$  mV for *bw; st* larvae, unpaired Student's *t*-test,  $P < 0.0001$ ). Heterozygous *rop<sup>A19/rop<sup>G11</sup></sup>* larvae showed a similar decrease in EJP amplitude when compared with controls ( $7.7 \pm 2.1$  mV, *t*-test,  $P < 0.001$ ). These decreases in EJP amplitude in the *rop<sup>G11/rop<sup>G11</sup></sup>* and *rop<sup>A19/rop<sup>G11</sup></sup>* larvae were observed at multiple calcium concentrations (0.6, 1.0 and 1.8 mM  $Ca^{2+}$ ) (Figure 4D and Table I).

In addition to defects in evoked response, these mutant larvae also displayed a reduction in the frequency of spontaneous fusion events in the absence of nerve stimulation (Figure 4C). *rop<sup>G11/rop<sup>G11</sup></sup>* larvae had an mEJP frequency of  $1.2 \pm 0.2$  mEJPs/s compared with  $3.1 \pm 0.5$  mEJPs/s in control larvae (unpaired Student's *t*-test,  $P < 0.001$ ). *rop<sup>A19/rop<sup>G11</sup></sup>* larvae showed a similar defect ( $0.9 \pm 0.1$  mEJPs/s, *t*-test,  $P < 0.001$ ). These defects in both evoked and spontaneous responses do not appear to be caused by developmental defects, since the morphology of *rop<sup>G11/rop<sup>G11</sup></sup>* and *rop<sup>A19/rop<sup>G11</sup></sup>* larvae appears normal at the light microscope level. To rule out the possibility that these physiological defects are due to alterations in synaptic morphology, we stained *rop<sup>G11/rop<sup>G11</sup></sup>* and *rop<sup>A19/rop<sup>G11</sup></sup>* third instar larvae with anti-synaptotagmin antibody, which specifically labels synapses (Littleton *et al.*, 1993a). The morphology and the number of synaptic boutons in these *rop* mutants were indistinguishable from wild-type (for *bw; st* larvae,  $2.3 \times 10^{-3} \pm 0.2 \times 10^{-3}$  boutons/ $\mu m^2$ ; for *rop<sup>A19/rop<sup>G11</sup></sup>*,  $2.3 \times 10^{-3} \pm 0.2 \times 10^{-3}$  boutons/ $\mu m^2$ , *t*-test,  $P = 0.85$ ; for *rop<sup>G11/rop<sup>G11</sup></sup>*,  $2.6 \times 10^{-3} \pm 0.2 \times 10^{-3}$  boutons/ $\mu m^2$ , *t*-test,  $p = 0.32$ ). Furthermore, the muscle resting potentials from *rop<sup>G11/rop<sup>G11</sup></sup>* and *rop<sup>A19/rop<sup>G11</sup></sup>* are similar to control larvae ( $-55.2 \pm 1.6$  mV in *rop<sup>G11/rop<sup>G11</sup></sup>* and  $-57.0 \pm 2.2$  mV in *rop<sup>A19/rop<sup>G11</sup></sup>* compared with  $-54.4 \pm 1.5$  mV in *bw; st* larvae) (Table I), and mEJP amplitudes are not altered measurably in control versus *rop<sup>G11/rop<sup>G11</sup></sup>* and *rop<sup>A19/rop<sup>G11</sup></sup>* larvae ( $0.71 \pm 0.04$  mV in *rop<sup>G11/rop<sup>G11</sup></sup>*,  $0.81 \pm 0.05$  mV in *rop<sup>A19/rop<sup>G11</sup></sup>* and  $0.73 \pm 0.01$  mV in *bw; st*) (Table I). Taken together, our results suggest that ROP function is required in the presynaptic boutons specifically for the regulation of both spontaneous and evoked neurotransmission.

#### **ROP plays an inhibitory role in neurotransmission**

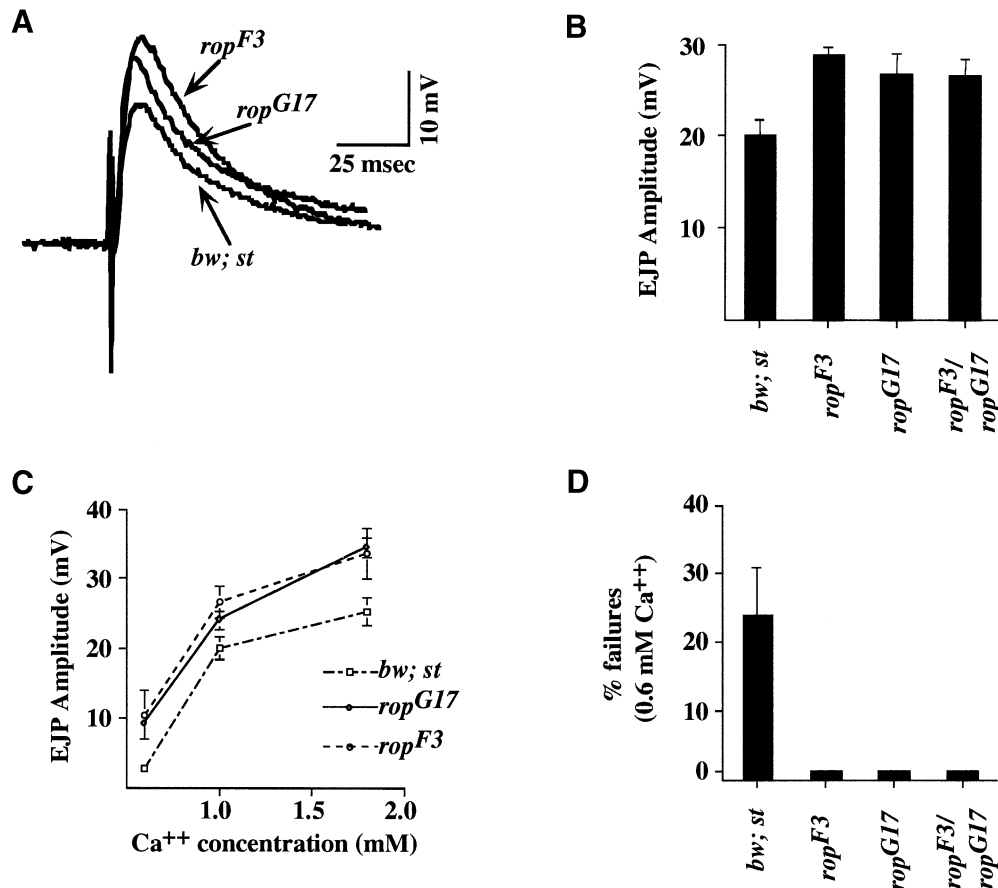
In addition to a positive role for the Sec1 family in secretion, an inhibitory role has also been proposed for n-Sec1 and ROP (Pevsner *et al.*, 1994a; Schulze *et al.*, 1994). ROP/n-Sec1 has been suggested to inhibit neuro-



**Fig. 4.** *rop<sup>A19</sup>* and *rop<sup>G11</sup>* mutations cause a decrease in evoked and spontaneous neurotransmitter release. (A) Representative EJPs are shown from control *bw; st* and homozygous *rop<sup>G11</sup>* and heterozygous *rop<sup>A19/rop<sup>G11</sup></sup>* larvae. These recordings were performed at 24°C and in 1 mM Ca<sup>2+</sup>. (B) Amplitude of evoked responses of *bw; st*, *rop<sup>G11</sup>* and *rop<sup>A19/rop<sup>G11</sup></sup>* larvae at room temperature and in 1 mM Ca<sup>2+</sup>. Average EJP amplitude are plotted, and error bars represent SEM. The EJP amplitude is reduced by 66% in homozygous *rop<sup>G11</sup>* larvae ( $n = 9$ ) compared with *bw; st*. Heterozygous *rop<sup>A19/rop<sup>G11</sup></sup>* larvae ( $n = 6$ ) show a 62% reduction compared with *bw; st*. (C) Frequency of spontaneous events in *bw; st*, *rop<sup>G11/rop<sup>G11</sup></sup>* and *rop<sup>A19/rop<sup>G11</sup></sup>* larvae. Average mEJP frequency is plotted, with error bars representing SEM. *rop<sup>G11</sup>* homozygous larvae ( $n = 9$ ) show a 64% decrease in frequency of spontaneous events compared with *bw; st*. *rop<sup>A19/rop<sup>G11</sup></sup>* larvae ( $n = 6$ ) display a 73% reduction in the number of miniature events. (D) Evoked responses of *bw; st*, *rop<sup>A19/rop<sup>G11</sup></sup>* and *rop<sup>G11/rop<sup>G11</sup></sup>* larvae over a range of Ca<sup>2+</sup> concentrations. EJPs were recorded in 0.6, 1.0 and 1.8 mM Ca<sup>2+</sup> in HL-3. EJPs are plotted against Ca<sup>2+</sup> concentration. Error bars represents SEM, and are shown only if the error is larger than the symbol. *rop<sup>G11</sup>* larvae show a reduced evoked response at the three Ca<sup>2+</sup> concentrations tested, with greater differences observed at the higher Ca<sup>2+</sup> concentrations. Similar results were obtained for *rop<sup>A19/rop<sup>G11</sup></sup>* larvae.

transmission by regulating syntaxin participation in the core complex. However, no direct *in vivo* evidence exists for such a role. In the analysis of *rop* mutations at the larval NMJ, *rop<sup>F3/rop<sup>F3</sup></sup>* and *rop<sup>G17/rop<sup>G17</sup></sup>* larvae revealed a different phenotype from that displayed by *rop<sup>G11/rop<sup>G11</sup></sup>* and *rop<sup>A19/rop<sup>G11</sup></sup>* larvae. As shown in Figure 5A and B, *rop<sup>F3/rop<sup>F3</sup></sup>* larvae showed a significant increase in EJP amplitude at room temperature when compared with control *bw; st* larvae ( $20.1 \pm 1.6$  mV in *bw; st* and  $28.7 \pm 0.9$  mV in *rop<sup>F3</sup>*,  $P < 0.01$ ). A similar increase in EJP

amplitude compared with control is also observed in homozygous *rop<sup>G17</sup>* larvae ( $26.6 \pm 2.3$  mV, *t*-test,  $P < 0.05$ ) (Figure 5A and B). This elevated EJP amplitude observed for *rop<sup>F3</sup>* and *rop<sup>G17</sup>* larvae is present at multiple calcium concentrations (Figure 5C and Table I) and represents an increase in evoked neurotransmitter release, because mEJP amplitude is not altered measurably in *rop<sup>F3</sup>* and *rop<sup>G11</sup>* mutants when compared with wild-type (Table I). To rule out the possibility that the enhanced evoked release that we observed in *rop<sup>F3</sup>* and *rop<sup>G17</sup>* homozygous larvae was



**Fig. 5.** *rop<sup>F3</sup>* and *rop<sup>G17</sup>* mutations cause an increase in evoked response. (A) Representative EJPs are shown for control *bw; st*, *rop<sup>F3</sup>/rop<sup>F3</sup>* and *rop<sup>G17</sup>/rop<sup>G17</sup>* larvae. Recordings were performed in 1.0 mM Ca<sup>2+</sup>. Stimulation artifact indicates onset of nerve stimulation. Scale bar: horizontal, 25 ms; vertical, 10 mV. (B) Average EJP amplitudes are plotted, with error expressed as SEM. Recordings were performed in HL-3 in 1.0 mM Ca<sup>2+</sup>. *rop<sup>F3</sup>/rop<sup>F3</sup>* larvae ( $n = 6$ ) display a 43% increase in evoked response, and *rop<sup>G17</sup>/rop<sup>G17</sup>* larvae ( $n = 8$ ) display a 32% increase when compared with controls. *rop<sup>F3</sup>/rop<sup>G17</sup>* larvae show a 32% increase, compared with controls. (C) Evoked responses at three Ca<sup>2+</sup> concentrations are plotted for *bw; st*, *rop<sup>G17</sup>/rop<sup>G17</sup>* and *rop<sup>F3</sup>/rop<sup>F3</sup>*. The error, when larger than the symbol, is expressed as SEM. *rop<sup>F3</sup>/rop<sup>F3</sup>* and *rop<sup>G17</sup>/rop<sup>G17</sup>* larvae display an increase in evoked response at these Ca<sup>2+</sup> concentrations. (D) Percentage of failures at 0.6 mM Ca<sup>2+</sup> in HL-3. Under low Ca<sup>2+</sup> conditions, no evoked response could be measured in control *bw; st* larvae upon stimulation 23.7% of the time (four larvae, 119 stimulations). In contrast, no failures were ever observed for either *rop<sup>F3</sup>/rop<sup>F3</sup>*, *rop<sup>G17</sup>/rop<sup>G17</sup>* or *rop<sup>F3</sup>/rop<sup>G17</sup>* larvae under these conditions (four larvae, minimum of 66 stimulations). Error is expressed as SEM.

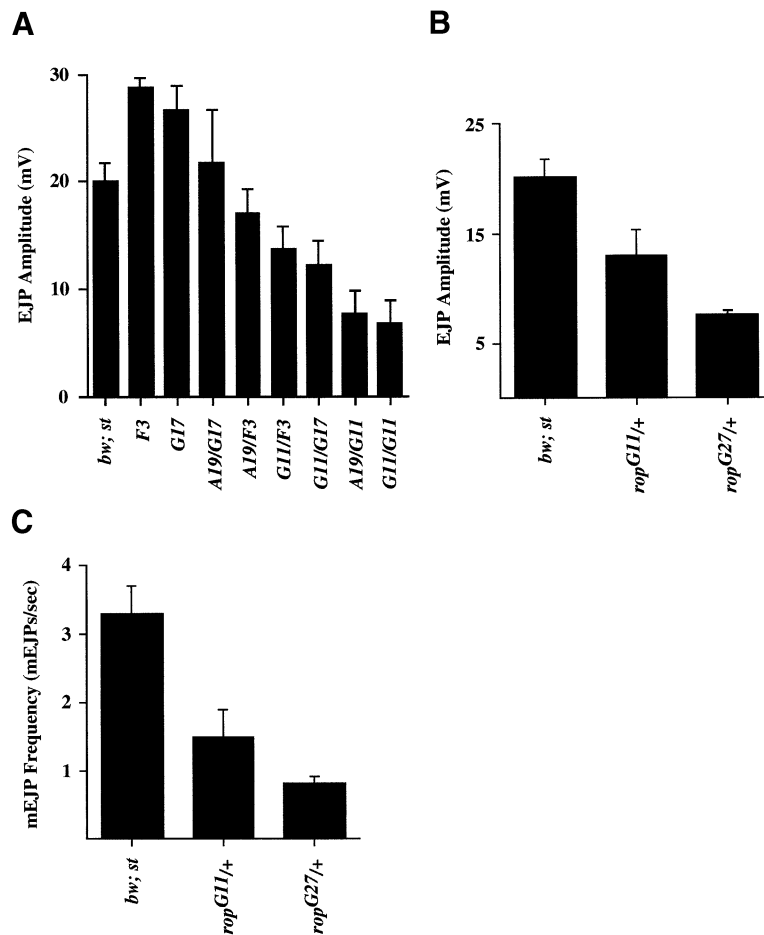
due to second site mutations, we analyzed *rop<sup>F3</sup>/rop<sup>G17</sup>* heterozygous animals. These animals showed a similar phenotype to *rop<sup>F3</sup>* and *rop<sup>G17</sup>* mutants at 0.6, 1.0 and 1.8 mM Ca<sup>2+</sup> (Figure 5B and Table I), indicating that second site mutations are not responsible for the enhanced evoked response observed in *rop<sup>F3</sup>* and *rop<sup>G17</sup>* mutants. As with *rop<sup>A19</sup>/rop<sup>G11</sup>* and *rop<sup>G11</sup>/rop<sup>G11</sup>* mutants, no significant changes in synaptic morphology or number were detected in *rop<sup>F3</sup>/rop<sup>F3</sup>* and *rop<sup>G17</sup>/rop<sup>G17</sup>* larvae with anti-synaptotagmin staining (for *bw; st* larvae,  $2.3 \times 10^{-3} \pm 0.2 \times 10^{-3}$  boutons/ $\mu\text{m}^2$ ; for *rop<sup>F3</sup>*,  $2.3 \times 10^{-3} \pm 0.3 \times 10^{-3}$  boutons/ $\mu\text{m}^2$ ,  $t$ -test,  $P = 0.87$ ; for *rop<sup>G17</sup>*,  $2.2 \times 10^{-3} \pm 0.06 \times 10^{-3}$  boutons/ $\mu\text{m}^2$ ,  $t$ -test,  $P = 0.93$ ).

At a low calcium concentration (0.6 mM Ca<sup>2+</sup> in HL-3), we observed a significant number of failures upon stimulation in control *bw; st* larvae ( $23.7 \pm 7.0\%$  of stimulations, four independent larvae, 119 stimulations). However, in homozygous *rop<sup>F3</sup>* and *rop<sup>G17</sup>*, as well as *rop<sup>F3</sup>/rop<sup>G17</sup>* larvae, no such failures were ever observed (four independent larvae, and a minimum of 66 stimulations) (Figure 5D), suggesting that the probability of release is increased in these mutants.

### Neurotransmission is highly sensitive to levels of ROP function

Characterization of heterozygous combinations of *rop* mutant alleles revealed that both evoked and spontaneous neurotransmitter release are highly sensitive to the levels of ROP function. On the one hand, the *rop<sup>A19</sup>* and *rop<sup>G11</sup>* alleles represent a class of mutations where neurotransmission is dramatically reduced. On the other hand, the *rop<sup>F3</sup>* and *rop<sup>G17</sup>* alleles represent another class which causes increased evoked release. Heteroallelic combinations between these classes result in intermediate phenotypes (Figure 6A). In general, these phenotypes are roughly the average of the effects of the two different alleles. For example, in 1 mM Ca<sup>2+</sup>, *rop<sup>F3</sup>* homozygous larvae show an EJP amplitude of ~28 mV, while *rop<sup>G11</sup>* homozygous larvae show an EJP amplitude of ~7 mV (Table I). *rop<sup>F3</sup>/rop<sup>G11</sup>* heterozygous larvae reveal an evoked response of 14 mV (Table I), which is close to the average of the responses for *rop<sup>F3</sup>/rop<sup>F3</sup>* and *rop<sup>G11</sup>/rop<sup>G11</sup>* mutants. Spontaneous neurotransmission is likewise sensitive to the different combinations of *rop* alleles (Table I).

Furthermore, defects in both evoked and spontaneous



**Fig. 6.** Neurotransmission is highly sensitive to levels of ROP function. (A) EJP amplitudes for control *bw; st* larvae and *rop<sup>F3</sup>*, *rop<sup>G17</sup>*, *rop<sup>A19</sup>/rop<sup>G17</sup>*, *rop<sup>A19</sup>/rop<sup>F3</sup>*, *rop<sup>G11</sup>/rop<sup>F3</sup>*, *rop<sup>G11</sup>/rop<sup>G17</sup>*, *rop<sup>A19</sup>/rop<sup>G11</sup>* and *rop<sup>G11</sup>/rop<sup>G11</sup>* larvae. Average EJP amplitudes are plotted with SEM as error. Different heterozygous combinations of *rop* alleles result in a gradient of intermediate phenotypes, underscoring the sensitivity of neurotransmission to levels of ROP function. (B) EJP amplitudes for control *bw; st* larvae and larvae bearing one wild-type copy of *rop* and either the *rop<sup>G11</sup>* or the *rop<sup>G27</sup>* allele. Average EJP amplitudes are plotted, with error bars representing SEM. Recordings were performed in 1 mM  $Ca^{2+}$ . The finding that one copy of *rop<sup>G11</sup>* or *rop<sup>G27</sup>* causes a dramatic reduction in evoked responses indicates that ROP is a rate-limiting component of neurotransmission. (C) Frequency of mEJPs for control *bw; st* larvae and larvae bearing one wild-type copy of *rop* and either the *rop<sup>G11</sup>* or the *rop<sup>G27</sup>* allele. Average mEJP frequencies are plotted and error is expressed as SEM.

responses are also found when only one copy of *rop<sup>G11</sup>* or the null allele *rop<sup>G27</sup>* is present. Heterozygous *bw; rop<sup>G11</sup> st/+ st* larvae were generated by crossing *rop<sup>G11</sup>/rop<sup>G11</sup>* flies with control *bw; st* flies. These heterozygous *bw; rop<sup>G11</sup> st/+ st* larvae showed a 35% reduction in EJP amplitude (Figure 6B, unpaired Student's *t*-test,  $P < 0.05$ ) and a 54% reduction in frequency of mEJPs (*t*-test,  $P < 0.05$ ) compared with *bw; st* controls (Figure 6C). In addition, heterozygotes generated between the null allele, *rop<sup>G27</sup>*, and *bw; st* demonstrate a 62% reduction in evoked response (Figure 6B, *t*-test,  $P < 0.001$ ) and a 79% reduction in mEJP frequency (Figure 6C, *t*-test,  $P < 0.05$ ) compared with *bw; st* controls. Heterozygotes containing one copy of the deficiency *Df(3L)GN34*, which removes the *rop* locus, also showed a significant decrease in neurotransmission when compared with *bw; st* controls, but these animals could not be compared quantitatively with other lines, because the deficiency was generated in a different genetic background (data not shown) (Harrison *et al.*, 1994). The finding that one mutant copy of either the *rop<sup>G11</sup>* allele or the null allele causes a dramatic decrease in EJP amplitude and mEJP frequency indicates that

ROP is a rate-limiting component of both evoked and spontaneous neurotransmitter release.

## Discussion

### Overexpression studies indicate that syntaxin and ROP interact in vivo

We have shown that overexpression of syntaxin leads to a decrease in both evoked and spontaneous neurotransmission. Interestingly, defects in secretion have also been observed in other systems when syntaxin-1A or Sed5 (the yeast endoplasmic reticulum–Golgi syntaxin homolog) are overexpressed in non-neuronal cells (Dascher *et al.*, 1994; Bittner *et al.*, 1996; Nagamatsu *et al.*, 1996). How might overexpression of syntaxin cause these effects? One possibility is that overproduction of syntaxin serves to titrate out other rate-limiting components of exocytosis. Another possibility is that overexpression of syntaxin may inhibit  $Ca^{2+}$  channel activity, as has been suggested by studies in other systems (Bezprozvanny *et al.*, 1995; Wiser *et al.*, 1996). However, since we also observe a concurrent decrease in the frequency of miniature events, which

are thought to represent spontaneous  $\text{Ca}^{2+}$ -independent quantal events,  $\text{Ca}^{2+}$  channel inhibition is unlikely to be the sole cause of the decrease in neurotransmitter release. It can be difficult to determine, solely on the basis of overexpression studies, the molecular defects underlying a phenotype. This difficulty is compounded by the observation that syntaxin binds many other proteins implicated in neurotransmitter release (Südhof, 1995). However, overexpression of SNAP-25, which binds all the same proteins that syntaxin binds (Hayashi *et al.*, 1994; Pevsner *et al.*, 1994a; Hanson *et al.*, 1995; McMahon *et al.*, 1995; Sheng *et al.*, 1996), with the exception of synaptotagmin, complexins and ROP/n-Sec1, does not inhibit neurotransmitter release. This observation suggests that overexpression of syntaxin inhibits exocytosis by an interaction outside the core complex. n-Sec1 has been shown to have the highest known affinity for syntaxin *in vitro* (Pevsner *et al.*, 1994a). Therefore, a simple interpretation of these findings is that overproduction of either syntaxin or ROP alone results in the excess of one component driving the formation of non-physiological amounts of syntaxin–ROP complex, which may inhibit neurotransmitter release by not allowing syntaxin (or ROP) to perform their required functions. However, when syntaxin and ROP are overexpressed simultaneously, we observe a suppression of these physiological defects. In this case, since there are roughly stoichiometric amounts of syntaxin and ROP, a sufficient population of syntaxin and ROP may exist that is not bound in a syntaxin–ROP complex, allowing both these proteins to perform their required functions. Regardless of the cause of the decreased neurotransmitter release observed when syntaxin or ROP is overexpressed alone, the finding that these defects are suppressed when syntaxin and ROP are overproduced simultaneously clearly indicates that these two proteins functionally interact *in vivo*. In addition, we find that overexpressing ROP, while causing a decrease in neurotransmission, does not cause a change in the number of docked vesicles at the synapse. This finding implies that at least some of the function of ROP is mediated after docking, and is consistent with a role for ROP in regulating syntaxin function, as syntaxin has been shown to be required for a post-docking role (Broadie *et al.*, 1995).

### **Two functions for ROP in exocytosis**

The four loss-of-function point mutations in ROP have allowed us genetically to dissect two distinct functions for the ROP protein in neurotransmitter release. It should be noted that the positions of these point mutations in ROP do not allow for an obvious division into distinct functional domains, suggesting that these point mutations affect the tertiary structure of ROP or that mutations with similar phenotypes affect a domain of ROP that consists of stretches of amino acids that are not contiguous in the primary structure. What is the required function of ROP? We show here that mutations in ROP can cause a variety of effects on evoked and spontaneous neurotransmission, suggesting that ROP may have multiple effector proteins *in vivo*. In addition to syntaxin, studies of yeast mutants have suggested that the Sec1 family may interact with Rab family members (Salminen and Novick, 1987; Dascher *et al.*, 1991). Rab proteins are Ras-like proteins that have been proposed to regulate nearly all stages of

membrane trafficking (Novick *et al.*, 1993). Recently, the vertebrate homolog of ROP, Munc-18, has been shown to biochemically interact with DOC2, a novel C2 domain-containing protein (Orita *et al.*, 1995; Verhage *et al.*, 1997). Therefore, the required function of ROP may be mediated by its interactions with different proteins.

In contrast to the *rop*<sup>G11</sup> and *rop*<sup>A19</sup> mutations which cause a dramatic decrease in neurotransmitter release, the *rop*<sup>F3</sup> and *rop*<sup>G17</sup> mutations cause a significant enhancement in evoked neurotransmission which was observed at different  $\text{Ca}^{2+}$  concentrations. Therefore, in these mutants, there is a defect in the inhibitory, but not the required, function of ROP. These data represent the first direct evidence for an inhibitory function for ROP *in vivo* and suggest that the mutated ROP in these lines may be unable to regulate normally the number of vesicles released following an action potential. We also observed a significant decrease in the failure rate in low release conditions, which suggests that either (i) the nerves of *rop*<sup>F3/rop</sup><sup>F3</sup> and *rop*<sup>G17/rop</sup><sup>G17</sup> mutants are more excitable or (ii) the probability of release is increased in these mutants. Excitability does not appear to be enhanced in these mutants since, at higher  $\text{Ca}^{2+}$  concentrations, multiple or prolonged EJPs are not observed in response to a single stimulus, as has been observed in mutants which affect nerve excitability (Jan *et al.*, 1977; Ganetzky and Wu, 1983). How could the probability of release be altered in these *rop* mutants? One possibility is that  $\text{Ca}^{2+}$  sensitivity is altered in these mutants. However, we do not observe such a change in the *rop* mutants analyzed. Another possibility is that there is an increase in the readily releasable pool of vesicles. The inhibitory function of ROP may therefore be to control the readily releasable pool of synaptic vesicles. This hypothesis is supported by the finding that *in vitro* the binding of n-Sec1 to syntaxin can inhibit syntaxin's binding to SNAP-25 and synaptobrevin (Pevsner *et al.*, 1994a), and suggests that the readily releasable pool may include vesicles for which core complexes have formed properly.

### **ROP is a rate-limiting component of neurotransmitter release**

Analysis of larvae that bear one wild-type copy of ROP in combination with either *rop*<sup>G11</sup> or a null allele, *rop*<sup>G27</sup>, indicates that ROP is rate-limiting for exocytosis. In both of these cases, significant decreases are observed in both evoked and spontaneous neurotransmitter release. In the case where half of ROP function is removed (*rop*<sup>G27/+</sup>), there is an ~50% decrease in the evoked response, indicating a linear relationship between ROP activity and neurotransmission. Spontaneous responses appear to be even more severely affected, although this may be because of a greater variability in these measurements. Analysis of heteroallelic combinations of *rop* mutant alleles also supports the hypothesis that neurotransmission is very sensitive to the levels of ROP function. This rate-limiting phenotype is, to our knowledge, unique among all the proteins implicated in neurotransmitter release (Littleton *et al.*, 1993b; DiAntonio and Schwarz, 1994; T.Littleton and K.Schulze, unpublished observations) and places ROP in a pivotal position in exocytosis, since any modulation of ROP levels, availability or activity could result in altered levels of both evoked and spontaneous neurotransmission.

Based on these and other findings, we propose that ROP is a key regulator of exocytosis. First, ROP can interact with syntaxin *in vivo*, placing this protein in close contact with a key component of the release machinery. Second, *in vitro* biochemical data suggest that such an interaction may regulate core complex formation. Third, ROP performs a required function in both evoked and spontaneous exocytosis. Fourth, ROP also normally functions to inhibit neurotransmitter release. Fifth, ROP function is absolutely rate-limiting for evoked and spontaneous neurotransmission.

One important question which arises from these studies is how ROP function is regulated? This question is particularly important in light of the finding that ROP is rate-limiting for neurotransmitter release. It has been reported that Munc-18/n-Sec1/rbSec1 can be phosphorylated by protein kinase C (PKC) *in vitro*, and this phosphorylation inhibits binding of Munc-18/n-Sec1/rbSec1 to syntaxin (Fujita *et al.*, 1996). In addition, UNC-18 can also be phosphorylated by PKC *in vitro* (Sassa *et al.*, 1996). Another potential means of regulating Munc-18/n-Sec1/rbSec1 function may be via nitric oxide (NO) (Meffert *et al.*, 1996). *In vitro*, NO increases formation of the core complex and decreases Munc-18/n-Sec1/rbSec1 binding to syntaxin. Both PKC and NO have been implicated in cellular models of learning and memory (for review, see Ben-Ari *et al.*, 1992; Schwartz, 1993; Larkman and Jack, 1995). Thus, two intracellular effectors that probably contribute to synaptic plasticity have been shown to regulate the syntaxin–ROP interaction. Future studies of the neuronal members of the Sec1 family and their regulation may further elucidate the processes that modulate neurotransmitter release and contribute to our understanding of cellular forms of learning.

## Materials and methods

### Molecular biology

The syntaxin cDNA (Schulze *et al.*, 1995) was cloned into the pHS-Casper vector (Pirrotta, 1988) and injected into embryos. One viable transformant line was obtained and another line was generated using ‘transposase hopping’ (Bellen *et al.*, 1989). A portion of the SNAP-25 open reading frame (Risinger *et al.*, 1993) was PCR amplified from genomic DNA, and genomic and cDNA libraries were screened with this PCR fragment. A full-length SNAP-25 cDNA clone was obtained and was cloned into the pHS-Casper vector. Several transformant lines were established.

### Fly stocks

All fly strains were raised at 24°C on standard cornmeal–molasses medium supplemented with live yeast. Overexpression lines used include *P{syx1}*, *P{syx2}*, *P{rop3}* (Schulze *et al.*, 1994) and two different *P{SNAP-25}* lines. *y w* was used to generate these lines and therefore serves as a control line. *P{syx1}–P{rop3}* was generated by crossing *P{syx1}* and *P{rop3}*. *P{syx2}–P{rop3}* was generated by crossing *P{syx2}* and *P{rop3}*. *rop* mutations used in this study include the loss-of-function alleles *rop<sup>A19</sup>*, *rop<sup>F3</sup>*, *rop<sup>G11</sup>* and *rop<sup>G17</sup>*, as well as the null allele *rop<sup>G27</sup>*, and *Df(3L)GN34* (Harrison *et al.*, 1994). *bw*; *st* was used as a control line for the *rop* mutations, because these mutations were generated in a *bw*; *st* genetic background.

### Protein extraction and protein immunoblots

Heat shock of larvae for Western analysis was performed at 37°C for 1 h on pre-warmed grape juice plates. After recovery for 1 h at 25°C, larval brains were dissected in 1× Jan’s Ringers solution (128 mM NaCl, 2 mM KCl, 4 mM MgCl<sub>2</sub>, 34 mM sucrose, 4.8 mM HEPES, pH 7.1) and placed directly in sample buffer. The brains were crushed, boiled for 5 min and then resolved by SDS–PAGE. The proteins

were transferred to nitrocellulose for immunoblotting. Visualization was achieved with enhanced chemiluminescence (Amersham). Quantitation of signals was performed using a Bio-Rad densitometer with exposures at which signals were linearly responsive to the dose. Anti-syntaxin (8C3) antibody was used at 1:1000 final concentration; anti-SNAP-25 antibody (71.1) was used at 1:1000; anti-ROP (2447) was used at 1:10 000.

### Electrophysiology at the third instar larval neuromuscular junction

Third instar larvae were grown at 24°C in uncrowded vials. Controls include *y w* for the overexpression lines and *bw*; *st* for the *rop* mutant alleles. For overexpression lines, larvae were either dissected directly or after 1 h heat shock at 37°C with 1 h rest at 25°C. *rop<sup>G11</sup>/rop<sup>G11</sup>* larvae were identified as non-Tubby larvae in crosses of *rop<sup>G11</sup>/TM6-Tubby* × *rop<sup>G11</sup>/TM6-Tubby*. Homozygous larvae carrying the *rop<sup>F3</sup>* or *rop<sup>G17</sup>* alleles were dissected directly. All heterozygous combinations of *rop* alleles were generated by crossing *rop allele#1/TM6-Tubby* with *rop allele#2/TM6-Tubby* and selecting non-Tb larvae.

Dissections, nerve stimulation and muscle recordings were performed essentially as described previously (Littleton *et al.*, 1993b). Recordings were performed in HL-3 (Stewart *et al.*, 1994) at either 1.5 mM (for the overexpression lines) or 0.6, 1.0 or 1.8 mM Ca<sup>2+</sup> (for *rop* mutant lines). Nerves innervating the ventral body wall muscles were cut near the ventral ganglion and were stimulated with a suction electrode. For intracellular muscle recordings, microelectrodes were pulled on a Flaming–Brown micropipette puller to tip resistances of ~10–20 MΩ and were filled with 3 M KCl. Nerves were stimulated for 0.1 ms at a voltage 1.5 times threshold voltage. Muscle 6 from abdominal segments 4–6 was used for data collection, with the majority of recordings performed from segment 4. One or two muscles were tested for each individual. mEJP frequency was measured for 15–30 s for each individual. Dissections and recordings were performed at 24°C. Data were analyzed with MacAdios II and Superscope systems from G.W. Instruments.

### Immunohistochemistry

*bw*; *st* and *rop* mutant third instar larvae were dissected in HL-3 (Stewart *et al.*, 1994) on a Sylgard plate with minuten pins. The larvae were dissected as described for electrophysiology. The samples were fixed in 4% formaldehyde for 20 min. Dsyt2 (Littleton *et al.*, 1993a) was used at 1:1000. The larvae were developed using the ABC kit from Vector Laboratories. The number of boutons on muscle fibers 6/7 in abdominal segment 4 were counted and corrected for muscle size as described in Schuster *et al.* (1996).

### Electron microscopy

All dissections were performed in saline (Broadie and Bate, 1993) and preparations fixed for 15–30 min with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2), washed briefly in respective buffer, incubated for 1 h in 1% osmium in dH<sub>2</sub>O, then for 5 min in dH<sub>2</sub>O, for 30 min in 2% aqueous solution of uranyl acetate, for 5 min in 70% ethanol, for 10 min in 100% ethanol, for 5 min in epoxypropane, and finally transferred to Araldite. Muscles 6 and 7 with labeled synapses were cut out with a razor blade splinter and embedded for sectioning from their dorsal side. The 50–80 nm sections (silver-grey to silver, Reichert–Jung Ultracut) were transferred to carbonated formvar-coated slot grids (Galey and Nilsson, 1966), post-contrasted for 5–10 min with lead citrate and analyzed on a Jeol 200CX. Random micrographs of synapses (defined by the presence of an active zone and extracellular material in the synaptic cleft; Broadie *et al.*, 1995) were taken. At each synapse, the length of sharp membrane stretches was measured, and vesicles were counted and classified as close to the membrane (≤15 μm) or docked (touching the membrane). Three animals of each genotype were analyzed, and measurements taken from 54 and 28 synapses with a total of 29.8 and 18.7 μm of analyzed membrane (data given in the sequence: *P{rop3}* with heat shock and *P{rop3}* without heat shock). For each synapse, the number of vesicles per analyzed membrane was calculated and the statistical significance determined by Mann–Whitney U-test. The error is presented as SEM. Micrographs were shuffled and the genotype was not known during the measurements.

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