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rop, a Drosophila Homolog of Yeast Sec1 and Vertebrate n-Sec1/Munc-18 Proteins, Is a Negative Regulator of Neurotransmitter Release In Vivo

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Summary

The mammalian homolog of the yeast Sec1p, n-Sec1/Munc-18 has been demonstrated to bind the presynaptic membrane protein syntaxin, a putative synaptic vesicle docking protein. To determine the role of n-Sec1/Munc-18 in neurotransmitter release in vivo, we have overexpressed the Drosophila homolog, rop, in third instar larvae and measured the electrophysiological consequences at the neuromuscular junction. A 3- to 5-fold induction of the rop protein causes a dramatic decrease in neurotransmitter release, suggesting rop may restrict the ability of vesicles to dock or of docked vesicles to fuse. Consistent with this hypothesis, rop overexpression also reduces the number of spontaneous vesicle fusions by more than 50%, and repetitive stimulation results in significant decreases in evoked responses similar to those observed in rab3a mutant mice. However, rop overexpression does not alter significantly the Ca^{2+} dependence of neurotransmitter release. We propose that the Drosophila n-Sec1/Munc-18 homolog plays a negative role in neurotransmitter release in vivo, in addition to its previously identified positive function, possibly by modulation of docking of synaptic vesicles or activation of a pre-fusion complex at the active zone.

Introduction

The molecular mechanisms underlying neurotransmitter release are currently the focus of intense investigation. Recently, more studies have focused on genetic approaches to study the role of proteins involved in neurotransmitter release in vivo (Geppert et al., 1994; Harrison et al., 1994; Littleton et al., 1994; Nonet et al., 1993). Biochemical purification of synapse-specific proteins from vertebrate brains and genetic analyses of secretory processes in yeast have revealed that some of the proteins required in neurotransmitter release have been evolutionarily conserved from yeast to human (Bennett and Scheller, 1993; Jahn and Südhof, 1994). This work provides a model for exocytosis at the synapse in which the synaptic vesicle–associated protein synaptobrevin/VAMP binds to a complex of proteins anchored in the presynaptic membrane that consists of syntaxin and SNAP-25 (Söllner et al., 1993). This complex may permit docking and/or form an activated complex of proteins (Hunt et al., 1994) that prepares the vesicle for fusion. Fusion of synaptic vesicles may then be accomplished or promoted by binding of the cytosolic factors NSF and α-SNAP (Söllner et al., 1993).

As release of neurotransmitter is a highly regulated exocytotic event, it is likely that several regulatory proteins act upon these complexes of proteins by specifically interacting with one or more proteins within the complex in a positive or negative fashion. These proteins could conceivably regulate events that would modify the number of docked vesicles, the number of vesicles released upon nerve stimulation, the size of the evoked response as a function of the intracellular Ca^{2+} influx, or the rate of synaptic vesicle recycling. At least one of these key proteins has been identified previously, as several experiments suggest that the synaptic vesicle–specific protein, synaptotagmin, regulates exocytotic fusion by acting as a Ca^{2+} sensor (Brose et al., 1992; Chapman and Jahn, 1994; Davletov and Südhof, 1993; Littleton et al., 1993b, 1994). Here, we describe the results of electrophysiological studies which suggest that the Drosophila homolog of UNC-18/Munc-18/n-Sec1 (Gengyo-Ando et al., 1993; Hata et al., 1993; Pevsner et al., 1994b; Garcia et al., 1994), named rop (Salzberg et al., 1993), is an important regulatory protein affecting neurotransmitter release in vivo. We have overexpressed the rop protein in Drosophila and examined the electrophysiological consequences of increased rop levels at neuromuscular junctions. Our data indicate that rop plays a negative regulatory role in neurotransmitter release in vivo. These results are in agreement with recent biochemical data that also suggest a negative modulatory role for n-Sec1 in neurotransmitter release (Pevsner et al., 1994a).

Results

Cellular and Subcellular Localization of the rop Protein

The *rop* gene has been previously cloned in Drosophila because it shares a bidirectional promoter with the *ras2* gene (Cohen et al., 1988; Salzberg et al., 1993). In situ hybridization experiments using *rop* and *ras2* cDNAs as probes indicate that both are expressed in the garland cells, but in addition, *rop* is expressed in the CNS (Salzberg et al., 1993). However, no immuno-

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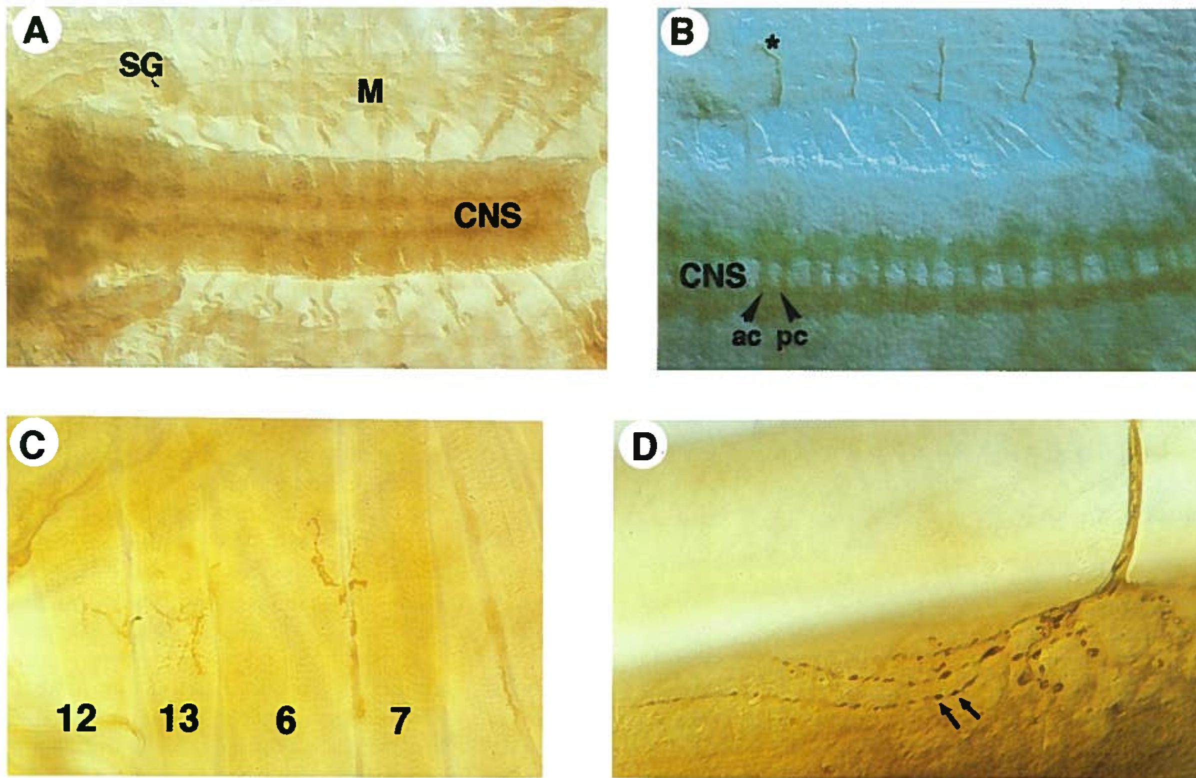

Figure 1. The rop Protein Is Expressed in Many Tissues and Is Present at Synapses

(A and B) Stage 16 filleted embryos (staging according to Campos-Ortega and Hartenstein [1985]) stained with anti-rop antibody. (A) Note expression of rop in the salivary glands (SG), muscles (M), and the CNS. (B) Detail of ventral nerve cord. Note expression of rop in the neuropil along the longitudinal tracts and, in addition, within the anterior and posterior commissures (ac, pc). The rop protein is also localized to muscle attachment sites (asterisks).

(C and D) Third instar larval neuromuscular junctions. (C) Synaptic localization of rop at terminals synapsing with ventrolateral muscle fibers 12, 13, 6, and 7. Muscle fiber 6 was routinely impaled in all electrophysiological recordings in this study. (D) rop protein is most abundantly present at neuromuscular boutons (indicated by arrows), but some protein is also present along the axons.

cytochemical localization of these proteins was presented. To determine the cellular and subcellular localization of rop, we have used an affinity-purified polyclonal anti-rop antibody prepared against a trpE–rop fusion protein. In the early stages of embryogenesis, we observe weak, relatively homogeneous rop expression throughout most of the embryo. At stage 13, a high level of rop expression is evident in the garland cell precursors, and the high degree of rop expression in these cells persists throughout embryonic development (data not shown). At stage 14, an increase in rop expression is observed in many tissues, including the CNS, the salivary glands, and the ectodermal cells. Later, in stages 15 and 16, rop protein is detected in muscles and muscle attachment sites (Figures 1A and 1B). As shown in Figure 1B, in the mature embryonic CNS, the rop protein is localized mainly in the neuropil along the longitudinal tracts where most synapses are located (Littleton et al., 1993a). In addition, rop protein is also present in the commissures, which consist mainly of axonal tracts. To confirm that the rop protein is localized to synapses, we immunocytochemically stained third instar larval fillets using the anti-rop antibody. As shown in Figures 1C and 1D, the rop protein is present in terminal axonal tracts at neuromuscular junctions and is highly abundant at boutons.

To determine the subcellular distribution of rop within neurons, we used Drosophila heads as a source to prepare various fractions enriched for synaptic vesicles and cytosolic proteins and fractions enriched in membranes (K. S., unpublished data). Aliquots of the various fractions were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and the identity and purity of each fraction were determined by Western blot analysis using a battery of synapse-specific antibodies. The affinity-purified anti-rop antibody recognizes a 67 kDa antigen that almost precisely matches the predicted molecular weight (Figure 2A). Since rop is essentially hydrophilic and contains no putative membrane-spanning domains, most of the rop protein was expected to be found in the cytosolic fraction. However, as shown in Figure 2A, the rop protein is most abundant in fractions enriched with membrane-associated proteins, such as syntaxin (K. S., unpublished data). rop is quite rare in association with unstripped vesicles and is completely absent in stripped vesicle fractions. These data suggest that

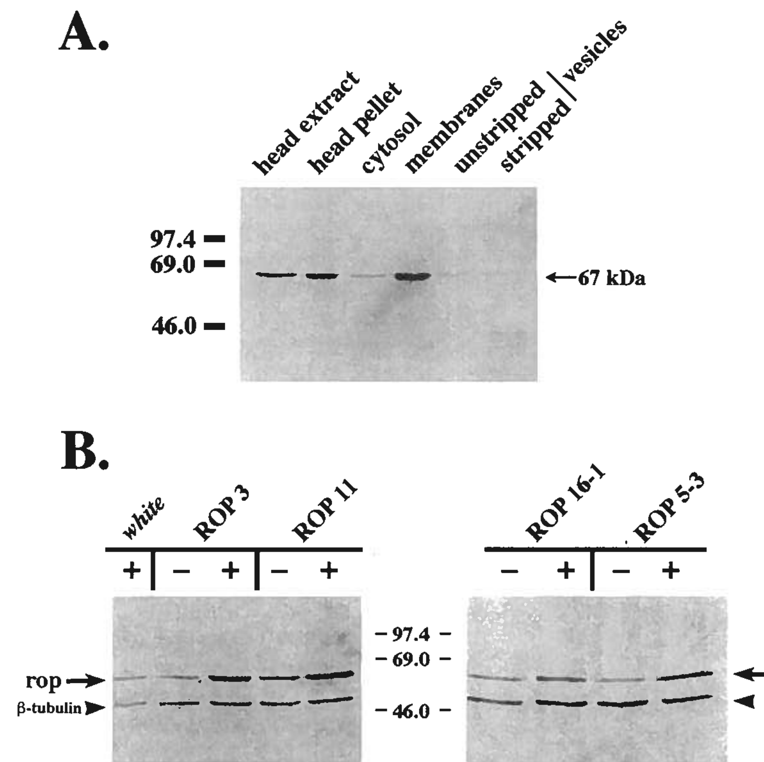

Figure 2. Subcellular Localization of rop and Overproduction of rop Protein in the CNS of Heat Shocked Larvae Bearing hsp70-*rop* Transgene

(A) Immunoblot of various fractions (15 μg of total protein loaded per lane) collected by sucrose step gradient centrifugation of a fly head extract. The rop protein is associated with membrane-enriched fractions derived from both the initial crude head pellet (lane 2) and the gradient pellet (membranes, lane 4). rop is also present in the cytosol (lane 3), but the protein is not associated with synaptic vesicles that have been stripped of associated proteins with a high pH sodium carbonate buffer (lane 6).

(B) Immunoblots of brains and ventral nerve cords dissected from *white*, ROP 3, ROP 11, ROP 16-1, and ROP 5-3 larvae. Each lane contains proteins of 5 third instar larvae that were maintained at 25°C (uninduced, −) or 5 larvae that were heat shocked for 1 hr at 37°C and processed after a 1.5 hr recovery period at 25°C (induced, +). Note that the CNS of ROP 3 and ROP 11 produce significantly more rop protein upon induction than ROP 16-1 and ROP 5-3, which show very little overexpression of rop in the CNS. These latter strains were therefore used as negative controls in subsequent electrophysiological analyses. The rop protein migrates at about 67 kDa (arrow). The 50 kDa band (arrowhead) corresponds to β-tubulin and serves as a control for amounts of protein loaded in each lane. Induction was quantified using the *GelScann(1.0)* algorithm (Metzker et al., submitted): heat shock of ROP 3 increases rop production by a factor of 4.9; heat induction of ROP 11 increases rop production by a factor of 3.3; heat shock of ROP 5-3 and ROP 16-1 induces rop overproduction by a factor of approximately 1.3.

rop is present at synapses in tight association with the neuronal membranes, analogous to the distribution of its vertebrate homolog (Pevsner et al., 1994b). The presence of rop at synapses suggests that rop may regulate neurotransmitter release in the fruitfly in a similar manner as proposed for n-Sec1/Munc-18 in mammals.

Electrophysiological Properties of Neuromuscular Junctions of Larvae That Overexpress rop

Absence of rop results in embryonic lethality in Drosophila (Harrison et al., 1994). In addition, the morphological defects in embryos that lack rop make patch clamping muscles very difficult (Kendal Broadie, personal communication). Hence, we are currently unable to address the electrophysiological consequences of loss of rop function at the neuromuscular junction. We therefore chose to address the function of rop in vivo by examining animals that overexpress rop, and hence to study the gain of function phenotype. The *rop* cDNA was subcloned in its sense orientation between the hsp70 5′ and 3′ promoter regions within the HS-REM3 heat shock vector (Knipple and Marsella-Herrick, 1988) in two parts: the partial cDNA containing the 5′ leader and the entire ORF, plus a genomic fragment that encompasses the 3′ untranslated region. The hsp70-driven *rop* transcription unit was subsequently cloned into a P element vector (Klemenz et al., 1987), and 15 independent transformants were obtained. The ability of these lines to overproduce rop protein upon a single 1 hr heat shock at 37°C was assessed 1.5 hr after heat treatment. As shown in Figure 2B, 2 lines (ROP 3 and ROP 11) were selected that demonstrated marked increases (5- and 3-fold, respectively) in rop production in the CNS of third instar larvae after heat shock, as well as 2 control insertion strains that induced rop very poorly (ROP 16-1 and ROP 5-3). Following heat shock of rop-inducible strains, higher levels of rop were detected in both the cytoplasmic and the membrane-associated fractions derived from adult fly heads. However, overexpression of rop did not affect the abundance of other synaptic proteins such as syntaxin, as determined by Western analysis (data not shown).

To test the effects of rop overexpression on synaptic physiology, third instar larvae were heat shocked for 1 hr at 37°C and allowed to recover for 1.5–2 hr at room temperature. Excitatory junctional potentials (EJPs) were elicited by nerve stimulation and recorded in both control and overexpressing lines. As shown in Figures 3a and 3b and Table 1, heat shocked ROP 3 and ROP 11 larvae demonstrated, respectively, a 45% and a 35% decrease in the amplitude of evoked neurotransmitter release at 1.0 mM extracellular Ca^{2+} com-

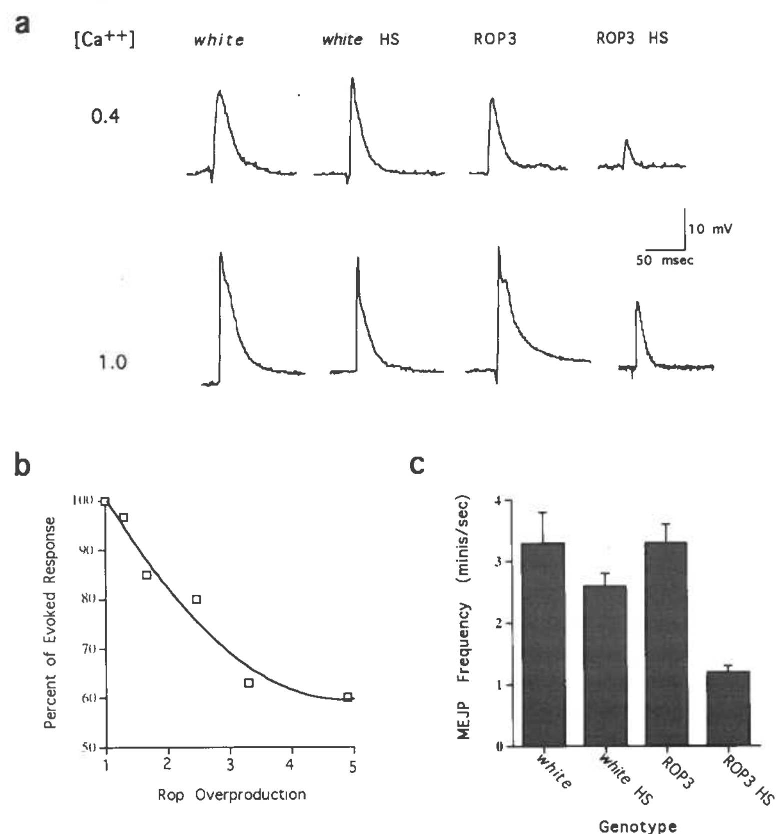

Figure 3. Synaptic Defects in rop-Overexpressing Lines

(a) Evoked responses in 3 control strains and 1 heat shocked rop-overexpressing line at the indicated external Ca^{2+} concentration (mM). The scale for the voltage trace is 10 mV; the scale for the sweep speed is 50 ms.

(b) Reduction of amplitude in evoked response in function of estimated rop overproduction. Note the strong negative correlation (Kendall rank = −1.0, $p < .02$) between the two variables.

(c) MEJP frequency measured in control and rop-overexpressing lines at 0.4 and 1.0 mM external Ca^{2+}. MEJP frequency did not change significantly in this $[Ca^{2+}]$ range. Control strains were *white* larvae (n = 7), the isogenized line into which the *rop*-containing vector was injected; *white* larvae that were heat shocked for 1 hr at 37°C and allowed to recover for 2 hr at room temperature (n = 16); and ROP 3 larvae that were not exposed to a heat pulse (n = 12). ROP 3 heat shocked larvae were exposed to 37°C for 1 hr and allowed to recover for 2 hr before recording (n = 12). HS, heat shocked. Error bars represent SEM.

pared with the uninduced and heat shocked control *white* larvae, as well as uninduced ROP 3 and ROP 11 larvae (unpaired Student's t test, $p < .01$). It should be emphasized that a 1 hr heat pulse followed by a 2 hr recovery period has no obvious effect on the electrophysiological properties of the neuromuscular junction of the *white* strain used for P element-mediated transformation, as no significant change in evoked responses or any other measured synaptic parameter is observed when compared with other wild-type strains (Table 1). To rule out any temperature-sensitive effects of wild-type *white* gene expression (which is very similar in all four strains) from the P element construct used for transformation, larvae of the ROP 5-3 and ROP 16-1 lines, which exhibit only a very subtle increase in rop (see above), were tested

Table 1. Electrophysiological Properties of ROP Heat Shock Lines

	Evoked Responses (mV)			MEJP Frequency	MEJP Amplitude
Strain	0.2 mM	0.4 mM	1.0 mM	(MEJPs/s)	(mV)
white	4.5 ± 0.8 (10)	20.2 ± 1.7 (6)	31.0 ± 1.9 (4)	3.3 ± 0.5 (7)	0.77 ± 0.04 (8)
white HS	3.5 ± 0.4 (5)	20.1 ± 1.6 (6)	26.9 ± 1.6 (11)	2.6 ± 0.2 (12)	0.78 ± 0.02 (15)
ROP 3	4.3 ± 1.2 (3)	20.8 ± 1.0 (6)	30.8 ± 2.7 (5)	3.3 ± 0.3 (12)	0.73 ± 0.04 (10)
ROP 3 HS	1.2 ± 0.3 (13)	7.9 ± 0.9 (9)	16.9 ± 2.3 (5)	1.2 ± 0.1 (12)	0.73 ± 0.02 (13)
ROP 3/+ HS	ND	ND	22.8 ± 1.2 (7)	2.4 ± 0.3 (6)	0.72 ± 0.01 (6)
ROP 3 HS+6hr	ND	ND	29.0 ± 1.0 (4)	3.2 ± 0.2 (4)	0.73 ± 0.01 (4)
ROP 11	ND	ND	27.5 ± 2.2 (5)	3.2 ± 0.4 (4)	0.70 ± 0.03 (5)
ROP 11 HS	ND	ND	17.9 ± 1.6 (14)	1.8 ± 0.4 (12)	0.71 ± 0.02 (12)
ROP 11/+ HS	ND	ND	24.4 ± 1.8 (5)	2.3 ± 0.2 (4)	0.72 ± 0.02 (4)
ROP 16-1	ND	ND	28.0 ± 2.1 (3)	3.2 ± 0.4 (3)	0.72 ± 0.02 (3)
ROP 16-1 HS	ND	ND	26.6 ± 1.5 (10)	2.8 ± 0.3 (10)	0.72 ± 0.02 (10)
ROP 5-3	ND	ND	27.0 ± 2.7 (8)	2.6 ± 0.3 (8)	0.74 ± 0.02 (8)
ROP 5-3 HS	ND	ND	28.2 ± 1.3 (8)	3.5 ± 0.3 (8)	0.75 ± 0.02 (8)

The number of larvae tested for each data point is indicated in parentheses. Errors are expressed as ± SEM. ND, not determined; HS, 1 hr 37°C heat pulse followed by 2 hr recovery period; HS + 6 hr, 1 hr 37°C heat pulse followed by 6 hr recovery period.

using the same paradigm. Again, no statistically significant defects were observed in these transformants compared with control larvae (Table 1).

If the overproduction of rop causes defects in evoked neurotransmitter release owing to its interference with some component of the release process, then the physiological effect of this interference should correlate with the levels of rop overproduction and should also exhibit saturation. As shown in Table 1 and Figure 3b, the synaptic defects in the ROP 3 line, which increase rop production by a factor of 4.9, are only slightly more severe than those caused by ROP 11, which increase rop production by a factor of 3.3. To confirm that the overproduction of rop indeed correlates with the reduction in neurotransmitter release, we determined the electrophysiological defects in ROP 3 and ROP 11 heterozygous larvae that each contain only a single heat shock rop construct, as opposed to homozygous larvae that each carry two copies of the construct. As shown in Figure 3b, ROP 3 and ROP 11 heterozygotes exhibit significantly less reduction in neurotransmitter release compared with homozygous ROP 3 and ROP 11 larvae. To verify that the overproduction of rop does not lead to a general cellular degeneration that would result in reduced responses, we examined synaptic physiology in ROP 3 heat shocked larvae that were allowed to recover for a full 6 hr before recording. Synaptic responses in these larvae had resumed pre-heat shock levels (see Table 1), demonstrating that rop overproduction does not cause irreversible defects in neurotransmitter release. It is also unlikely that overexpression of a synaptic protein simply reduces neurotransmitter release in a nonspecific manner, owing to its increased abundance, as a 10-fold overexpression of the presynaptic protein frequenin induced by heat shock actually increases neurotransmitter release (Rivosecchi et al., 1994). We therefore conclude that an overproduction of rop significantly and specifically reduces the amplitude of evoked synaptic transmission, consistent with a saturable interference with some component of the release pathway.

The decreases in evoked release could be attributed to a number of synaptic changes, including a decrease in the number of docked vesicles, a decrease in the activation of docked vesicles, a block in the fusion of synaptic vesicles with the presynaptic membrane, a decrease in the vesicle pool size, a change in the Ca^{2+} dependence of neurotransmitter release, or a change in muscle properties of rop-overexpressing lines. To differentiate between these possibilities, a number of additional synaptic parameters were examined. If spontaneous vesicle fusions (measured as miniature excitatory junctional potentials [MEJPs]) are due to the fusion of docked and activated vesicles with the presynaptic membrane, and if there is a reduction in the number of docked and/or activated synaptic vesicles at the active zone in the presence of excess rop, one would expect a corresponding decrease in the frequency of MEJPs. As shown in Figure 3c and Table 1, MEJP frequency was reduced by 64% in ROP 3 heat shocked larvae (unpaired Student's t test, $p < .001$) and 45% in ROP 11 heat shocked larvae (unpaired Student's t test, $p < .03$), whereas heat shocked *white*, ROP 5-3, and ROP 16-1 MEJP frequencies were not significantly different statistically from uninduced controls. MEJP amplitude, however, was not altered significantly among all the strains tested (Table 1). In addition, both overexpressing and control strains have resting potentials of about −50 mV (data not shown). As MEJP amplitude and muscle resting potentials are not affected in any of the strains tested, we conclude that muscles in rop-overexpressing lines respond normally to released neurotransmitter and that the decreases we observe in evoked responses and MEJP frequency are of presynaptic origin. To determine whether there were any obvious changes in the size of the synaptic vesicle pool after rop overproduction, we stained ROP 3 larvae that were given a 1 hr heat pulse and a 2 hr recovery with antibodies prepared against Drosophila synaptotagmin, a well-characterized marker for synaptic vesicles (Littleton et al., 1993a). Lines overexpressing rop showed no obvious changes in the distribution or intensity of synaptotagmin staining at neuromuscular junctions compared with controls (data not shown). These data suggest that there is no obvious change in the synaptic vesicle pool that can be detected at the light microscopic level. Thus, our data indicate that rop may play a negative regulatory role in vesicle docking or the activation of docked vesicles.

To assess any Ca^{2+} dependence of the reduction of evoked neurotransmitter release upon excess rop, ROP 3 induced larvae were tested over a range of external $[Ca^{2+}]$. As shown in Figure 4a, rop overproduction significantly reduced neurotransmitter release at all Ca^{2+} concentrations tested. However, with increases in $[Ca^{2+}]$, the evoked responses of ROP 3 induced larvae increased in a manner similar to that of controls. Thus, it is unlikely that rop overexpression affects the Ca^{2+} dependence of neurotransmitter release. To test this hypothesis directly, the Ca^{2+} dependence of neurotransmittter release was tested in control and ROP 3 heat-induced strains (Figure 4b). Dodge and Rahaminoff (1967) first measured the Ca^{2+} dependence of neurotransmitter release and found a fourth order relationship between extracellular $[Ca^{2+}]$ and the amplitude of the EJP. It was concluded from these and other studies that three to four calcium ions are required in a cooperative action to evoke release. Both heat shocked ROP 3 and *white* larvae have a Ca^{2+} dependence of neurotransmitter release (n) between 3.0 and 3.4. Overexpression of rop shifts the curve to the right and reduces the mean quantal content at a given $[Ca^{2+}]$ but does not alter significantly the order of the Ca^{2+} dependence of neurotransmitter release, indicated by the slope or n. This is in sharp contrast to the effect of some *synaptotagmin* mutations, which reduce the order of the Ca^{2+} dependence of neurotransmitter release from 3.5 to 1.6 (Littleton et al.,

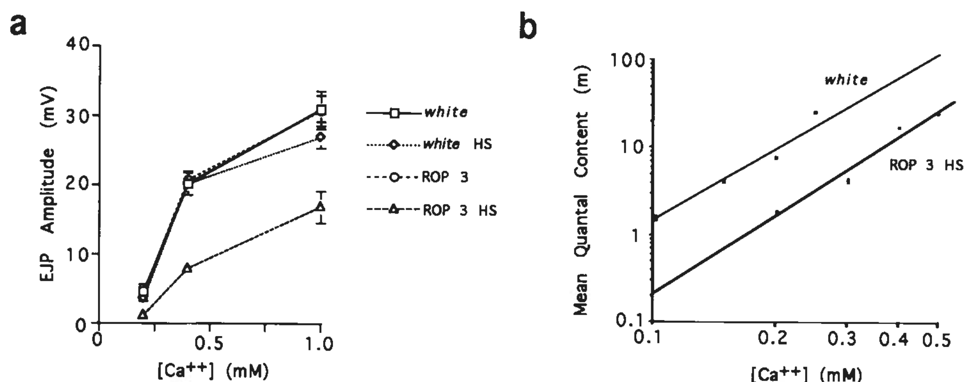

Figure 4. Evoked Responses at Varying External [Ca^{2+}]

(a) EJP amplitude for control and ROP 3-overexpressing larvae plotted over the range of [Ca^{2+}] examined. Measurements represent responses from 3–11 larvae of each genotype as shown in Table 1. Error bars represent SEM and are shown where larger than symbol. (b) Ca^{2+} dependence of neurotransmitter release in control *white* and ROP 3 heat shocked larvae. *white* larvae have slope values (n) for the double log plot of mean quantal content (EJP/MEJP) versus [Ca^{2+}] of approximately 3.4, whereas ROP 3 heat shocked larvae have an n value of 3.0. Error bars are SEM and are shown where larger than symbol. Correction of quantal values was as previously described (Martin, 1955).

1994). Given that the Ca^{2+} dependence of release is not affected significantly in the presence of additional rop, our data suggest that the defect in evoked response is unlikely to be due to defects in the Ca^{2+} activation of fusion of synaptic vesicles with the presynaptic membrane. Hence, the simplest interpretation is that rop negatively controls the number of docked vesicles at the active zone or interferes with the activation of the pre-fusion complex.

If rop indeed interferes with vesicle docking or activation of the pre-fusion complex, high frequency stimulation would result in a gradual depletion of the number of vesicles available for release, and hence, a further reduction in EJP amplitude. To test this hypothesis, control and rop-overexpressing strains were stimulated at 10 Hz for 30 s. Of the 20 control larvae tested (*white*, heat shocked *white*, and uninduced ROP 3 and ROP 11), 12 showed no decreases in EJP amplitude after 30 s. In contrast, 13 of 15 overexpressing larvae (heat shock–induced ROP 3 and ROP 11) demonstrated decreases in evoked responses after 30 s. Figure 5 shows representative traces from *white* and heat shocked ROP 3 larvae. The average decrease in controls was 3.6% ± 1.1% compared with a decrease of 27.2% ± 4.3% in rop-overexpressing lines. These differences are statistically significant (Mann-Whitney $U = 262$, $n_1 = 20$, $n_2 = 15$, $p < .001$) and suggest a reduction in the number of vesicles available for release upon repeated stimulation. Interestingly, further stimulations at 10 Hz did not reduce the amplitude of the ensuing EJPs beyond the initial decreases observed. These results suggest that vesicle recycling is not affected upon high frequency stimulation beyond an initial decrease in evoked response, and that it is unlikely that rop overproduction may non-specifically aggregate vesicles, making them unavailable for release. It remains to be established whether the rop protein has no access to actively recycling vesicles, whether there are multiple mechanisms for docking that bypass rop, or whether the levels of rop overproduction are insufficient to deplete the vesicle pool completely.

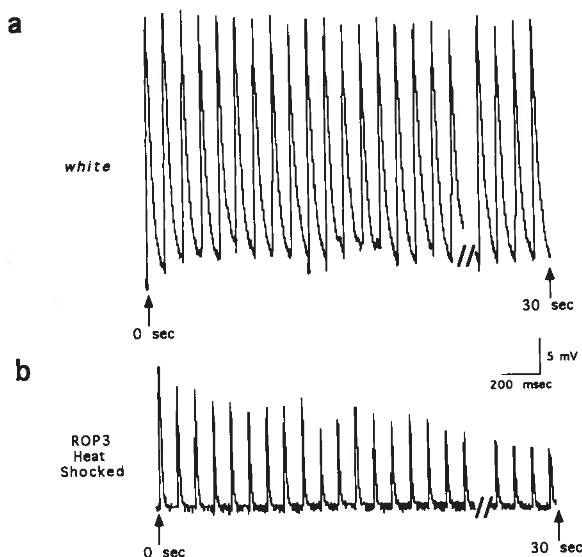

Figure 5. ROP Overexpression Results in a Decrease of Neurotransmitter Release during Repetitive Stimulation

EJPs from a *white* larva (a) or ROP 3 heat shocked larva (b) recorded in 1.0 mM external Ca^{2+} at 10 Hz stimulation frequency. Voltage scale is 5 mV; time scale is 200 ms. Of 15 ROP 3 and ROP 11 heat shocked larvae, 13 showed decreases of 27.2% ± 4.3% after 30 s of repetitive stimulation. Only 8 of 20 control larvae exhibited decreases, with an average of 3.6% ± 1.1%.

Another observation suggesting a negative role for rop in neurotransmitter release was made at low [Ca^{2+}] (0.2 mM). Repetitive stimulation at high frequency in low [Ca^{2+}] is known to cause an increase in neurotransmitter release. This synaptic property is termed facili-

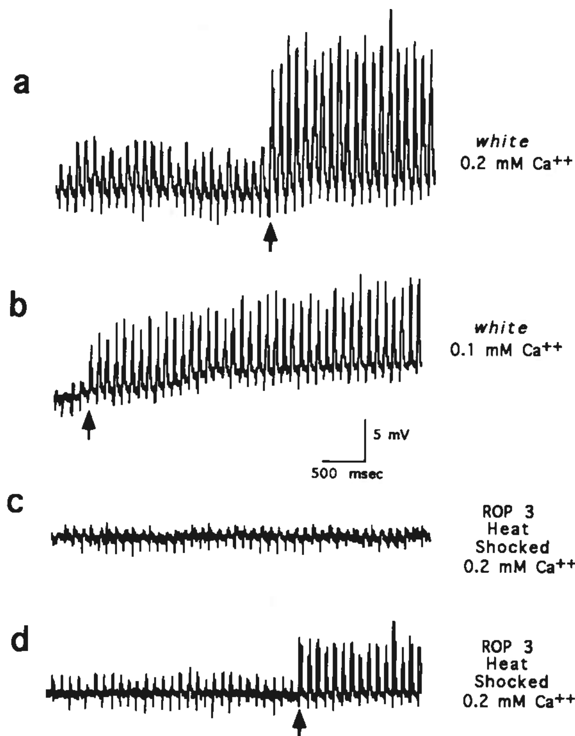

Figure 6. rop Overexpression Results in Decreased Synaptic Facilitation at Low [Ca^{2+}]

Stimulation (10 Hz) in *white* larvae at 0.2 mM external Ca^{2+} (a) or 0.1 mM external Ca^{2+} (b) and in ROP 3 heat shocked larvae at 0.2 mM external Ca^{2+} (c and d). Voltage scale is 5 mV; time scale is 500 ms. All control larvae (n = 12) showed synaptic facilitation after 5.0 ± 1.4 s. Of 14 ROP 3 larvae, 7 showed no facilitation (c), whereas the remaining 7 showed facilitation in 11.4 ± 3.3 s (d). The average facilitation in controls at 0.2 mM Ca^{2+} was 502% ± 96%, compared with 262% ± 103% in ROP 3 heat shocked larvae. These differences are not due to the smaller starting response in ROP 3 heat shocked larvae compared with controls, as *white* larvae at 0.1 mM Ca^{2+}, in which the mean quantal content is comparable to ROP 3 heat shocked larvae at 0.2 mM Ca^{2+}, demonstrate robust synaptic facilitation (b) with even greater average increases in synaptic responses.

tation and has been suggested to be due to a build-up of intracellular Ca^{2+} in the nerve terminal (Katz and Miledi, 1967). Wild-type larvae typically exhibit facilitation at 0.2 mM and lower [Ca^{2+}]. All 12 control larvae tested exhibited facilitation at 10 Hz, whereas only 7 of 14 ROP 3-overexpressing larvae exhibited some degree of facilitation (Figures 6a, 6b, and 6c; Mann-Whitney U = 130, n_1 = 14, n_2 = 12, $p < .01$). As shown in Figure 6d, those heat shocked ROP 3 larvae that demonstrated an increased evoked response exhibited facilitated EJPs that were significantly reduced in amplitude when compared with controls (ROP 3, 262% ± 103%; control, 502% ± 96%). In addition, the ROP 3 induced larvae that did undergo facilitation required on average significantly longer trains of 10 Hz stimulation than controls to elicit facilitated responses (11.4 ± 3.3 s compared with 5.0 ± 1.4 s; unpaired Student's t test, $p < .05$). These results are again consistent with a decreased number of docked or activated vesicles at the active zone, leading to a subsequent inability or reduced ability to generate facilitated responses typically observed after high frequency stimulation in low [Ca^{2+}].

Discussion

Mutational analysis of the Drosophila *rop* gene has shown that the rop protein is essential for imaginal cell viability, that it plays a key role in secretion in many embryonic tissues and cells, and that neurotransmitter release in adult photoreceptor cells is severely impeded in the absence of rop (Harrison et al., 1994). Similar, although less severe, defects have been observed in mutants of the *rop* homolog *unc-18* in Caenorhabditis elegans (Gengyo-Ando et al., 1993). The observation that mutations in *rop* cause a severe disruption of embryonic development beyond stage 14 precludes an electrophysiological analysis of embryos that lack rop (Kendal Broadie, personal communication), and intracellular recordings in C. elegans cannot be performed at present. We therefore initiated the studies presented here, which are aimed at understanding the role of rop in vivo by producing a gain of function phenotype through overexpression of the protein in third instar larvae.

Before initiating an electrophysiological analysis, we determined where the rop protein is expressed and, particularly, whether the rop protein is present in neurons at synapses. We find that the rop protein is expressed in most ectodermal cells, the garland cells, muscles, muscle attachment sites, the CNS, and the salivary gland cells. Interestingly, most of the rop protein in the CNS is localized to the embryonic neuropil, where most CNS synapses are localized (Littleton et al., 1993a). However, staining can also be observed in the commissures of the CNS, which mainly correspond to axonal tracts. Hence, rop is localized both to synapses and axons of the embryonic CNS, and a similar distribution is observed in third instar larvae.

To determine the subcellular localization of rop at synapses, we used a fractionation protocol (K. S., unpublished data) that allows us to establish within which subcellular compartments rop is concentrated. rop is most abundant in those fractions enriched for membrane or cytosolic proteins but is essentially absent from synaptic vesicle fractions. As rop does not have any noticeable transmembrane-spanning domain, we propose that its presence in the membranous fraction is due to its tight association with syntaxin-1a (Garcia et al., 1994; Hata et al., 1993; Pevsner et al., 1994b). Since significantly more rop protein seems to be associated with the membrane fraction compared with the cytosolic fraction, most of the rop protein may be bound to syntaxin-1a at the synapse, in agreement with our observation that syntaxin-1a

binds tightly to the rop protein in vitro (Halachmi et al., 1994, J. Neurochem., abstract).

Overexpression of rop does not affect several properties of neuromuscular junctions that we have investigated. First, the resting potential of muscles is not altered by overexpression of rop, and the amplitude of the MEJPs is identical in control and heat shocked ROP lines. Hence, it is most likely that the defects we observe are due to a presynaptic and not a postsynaptic defect. Second, we observe no significant change in the order of the Ca^{2+} dependence of neurotransmitter release, as heat shock–induced ROP and control larvae exhibit an n of 3.0 and 3.4, respectively. We therefore conclude that the rop protein is probably not involved in Ca^{2+} sensing, in contrast to a proposed function for synaptotagmin (Brose et al., 1992; Littleton et al., 1993b, 1994).

Interestingly, overexpression of rop alters key electrophysiological properties: first, we observe a very significant decrease in evoked response, and second, the frequency of MEJPs is decreased by up to 70%. These observations clearly indicate a negative regulatory role for rop in neurotransmitter release. Given the subcellular distribution of rop and its association with syntaxin-1a in Drosophila (Halachmi et al., 1994, J. Neurochem., abstract), it is likely that rop interferes with the function of Drosophila syntaxin-1a, which has been recently cloned (K. S., unpublished data). Since syntaxin-1a has been implicated in docking, we propose that overexpression of rop, through its interaction with syntaxin, reduces the number of available synaptic vesicles for fusion by inhibiting docking of individual vesicles. This model is supported by our finding that the defects in rop-overexpressing larvae correlate well with the amount of rop overproduction, consistent with a saturable interaction with a component of the release process such as syntaxin-1a. This conclusion is also supported by independent biochemical experiments which suggest that binding of n-Sec1 to syntaxin-1a inhibits SNAP-25 binding, producing a nonfunctional membrane receptor to which synaptobrevin/VAMP binds inefficiently (Pevsner et al., 1994a). Alternatively, rop may interfere with a step between vesicle docking and fusion. Hunt et al. (1994) have provided evidence that synaptobrevin plays a key role in this intermediate step and that synaptobrevin may not be involved in docking. Hence, rop could also interfere negatively with the function of this intermediate step(s). Regardless, our observations have important implications, as they indicate that MEJPs indeed represent the spontaneous fusion of properly docked vesicles at the active zone.

The conclusion that rop plays a negative role by inhibiting docking of synaptic vesicles or formation of a post-docking, pre-fusion complex is further supported by two independent observations. First, repetitive stimulation (10 Hz) at high extracellular [Ca^{2+}] leads to a gradual decrease in the amplitude of the evoked response in heat shocked larvae. This synaptic depression is strikingly similar in profile and relative amplitude to the synaptic depression observed in mice that lack the rab3a protein. The rab3a protein is a nonessential protein required for the efficient recruitment of synaptic vesicles for exocytosis during repetitive stimulation (Geppert et al., 1994). The similarity of the lack of function phenotype of rab3a mutants with the gain of function phenotype generated by the overexpression of rop can be explained easily with a simple model which assumes that rab3a modulates and/or displaces binding of rop to syntaxin (Pevsner et al., 1994a). Hence, when rop is present at a higher concentration than normal, rab3a activity is quickly exhausted, and when rab3a is absent, rop's negative impact is intensified, such that both situations lead to synaptic depression. Indeed, Dascher et al. (1991) have proposed an interaction between the yeast rab homolog Ypt1p and the rop-like Sly1p based on the ability of a single copy of *SLY1-20* to suppress the loss of *YPT1* function. This *SLY1* allele bears a single amino acid change which may allow it to assume a conformation that permits subsequent pre-fusion steps even in the absense of Ypt1p. Wild-type Sly1p may adopt this conformation only after it has been acted upon by Ypt1p. Because rab3a is known to be expressed exclusively in the PNS and CNS in Drosophila (K. S., unpublished data), similar mechanisms may regulate exo- or endocytosis in other tissues by utilizing other small GTP-binding proteins, such as ras2, which is coexpressed with rop in the garland cells (Salzberg et al., 1993).

Second, repetitive stimulation at low extracellular [Ca^{2+}] leads to absent or reduced amplitude facilitation in ROP-overexpressing lines when compared with controls. This observation confirms our previous data, if one assumes that fewer docked or activated vesicles are available for release. However, given the overall severity of defects in *rop* null mutations, it is unlikely that rop's only role in neurotransmission would be to interfere with the docking of vesicles. In fact, the lack or exhaustion of on- and off-transients in rop transheterozygous loss of function alleles indicates that rop has a positive function in the regulation of vesicle availability or of vesicle docking (Harrison et al., 1994). To this end, we propose a model in which rop's interaction with syntaxin (and possibly additional components of the release process) plays a positive role in secretion, possibly through an activation of the docking–pre-fusion complex. An activated rop–syntaxin complex would then require an additional level of modulation before syntaxin could become available for interactions with synaptobrevin and SNAP-25, eventually facilitating vesicle fusion. The disruption of an activated syntaxin–rop complex might well require an energy input, given the strong in vitro interactions reported between the two proteins (Pevsner et al., 1994a, 1994b). Small GTP-binding proteins, like rab3A, could provide the necessary energy required for separation and cycling of the rop–syntaxin complex. By overexpressing rop, it is likely that we have altered such a modification, thus unmasking

a negative role for an undisrupted rop–syntaxin complex in secretion. Given the dramatic reduction in neurotransmitter release owing to rop overproduction, it is quite likely that in vivo modulations of a rop–syntaxin interaction could be critical for the regulation of synaptic function and plasticity. In conclusion, the effect of rop overexpression on neurotransmitter release in vivo, as well as corroborating biochemical data (Pevsner et al., 1994a), collectively indicate that rop's interaction with a protein complex at the presynaptic membrane has the ultimate effect of controlling either the quantity of docked vesicles or the number of vesicles available for release at the active zone.

Experimental Procedures

Immunohistochemistry

Embryos were stained essentially as described by Salzberg et al. (1994) with anti-rop antibody that was affinity purified against recombinant rop protein. The final concentration of this antibody was 1:200. For Figures 1A and 1B, the embryos were filleted prior to photography. Staining of the neuromuscular junction was performed as described in Littleton et al. (1993a) using a final concentration of antibody of 1:500.

Cell Fractionation, Synaptic Vesicle Preparation, and Protein Immunoblots

Preparation of the various cell fractions was performed (K. S., unpublished data) from 5 ml of fly heads frozen in liquid nitrogen, finely ground with a mortar and pestle, suspended in 0.2 M sucrose, and homogenized. After a brief 10,000 rpm centrifugation to pellet cuticle and debris (head pellet), the supernatant was applied to a sucrose gradient composed of 0.4 M and 0.2 M sucrose steps. After a 2 hr centrifugation at 22,000 rpm, the supernatant (cytosol), 0.2–0.4 M interface (synaptic vesicles), and gradient pellet (neuronal membranes) were removed, and the latter was resuspended in a 10 mM HEPES (pH 7.4), 150 mM NaCl buffer. Synaptic vesicles were further treated for 30 min on ice with 100 mM Na_2CO_3 (pH 11.0) (stripped synaptic vesicles). All solutions were supplemented with protease inhibitors, and manipulations were performed at 4°C.

Proteins from each of the representative fractions were resolved by SDS–PAGE and transferred to nitrocellulose. Affinity-purified anti-rop antibody was utilized at a final dilution of 1:10,000, and detection was achieved with enhanced chemiluminescence (Amersham).

Heat shock of larvae for immunoblot analysis was performed in a 37°C incubator for 1 hr on prewarmed grape juice–agar plates. After 1.5 hr recovery at 25°C, five brains were dissected from larvae of each strain, crushed in 4× Laemmli buffer, boiled at 100°C for 5 min, and spun briefly in a microcentrifuge. Proteins were resolved on SDS–polyacrylamide gels and transferred to nitrocellulose for immunoblotting as described above. Anti-rop affinity-purified antibody was utilized at a final dilution of 1:5000, and monoclonal anti-β-tubulin antibody (Amersham) was used at a final concentration of 1:1000.

Molecular Biology

The *rop* cDNA (Salzberg et al., 1993) was cloned in pHSREM-3 vector under the regulation of the hsp70 promoter (Knipple and Marsella-Herrick, 1988) and subsequently cloned into the P[w8]-D vector (Klemenz et al., 1987), a derivative of P[w8]. Standard cloning procedures were used as described by Sambrook et al. (1989). The P[w8]-D-hsrop construct was injected into embryos and 2 homozygous viable transformant lines that carry the transgene in different chromosomal loci were established. Using "transposase hopping" (Bellen et al., 1989), 13 additional insertions were recovered.

Electrophysiology at the Third Instar Neuromuscular Junction

Dissections, nerve stimulation, and intracellular muscle recordings were performed as previously described (Jan and Jan, 1976; Littleton et al., 1994). Nerves that innervate the body wall muscles were cut and stimulated with a suction electrode. Nerves were stimulated for 0.1 ms at a voltage 1.5 times the threshold voltage. Muscle 6 from abdominal segments 4 and 5 was used for data collection. Data were analyzed with the MacAdios II and Superscope systems from G. W. Instruments.

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