medial γ lobe might fulfill a similar role to adult vertical projections in long-term information processing. Loss of these projections during metamorphosis could erase some long-term information specific to the larval stage. In adult *Drosophila*, few efferent neurons from the γ lobe extend to around the α α′ lobes (*12*), which could represent a pathway that converts information from short-term to long-term memory. Alternatively LTM may form independently of short-term memory. Further analyses must be performed to resolve that issue and to determine the role of α and α′ lobes in LTM formation.

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19. The wild-type reference stock was Canton-Special (CS). The *ala*^{1} P-element induced mutant (abbreviated *ala*) and the *ala*^{E13} excision mutant (*14*) were outcrossed in a CS background to prevent modifier accumulation. Flies were conditioned by exposure to one odor paired with electric shocks and subsequent exposure to a second odor in the absence of shock. A barrel-type machine was designed that allowed simultaneous automated conditioning of six groups of flies (*20*). During the tests, flies were exposed simultaneously to both odors in a T-maze (*21*). After 2 min, flies were trapped in either T-maze arm and counted. A reciprocal conditioning experiment was run with different flies of the same genotype, the second odor being associated with electric shock. The performance index (PI) corresponds to an averaged and normalized probability of the correct answer, so that a 50:50 distribution (no memory) yields a PI of zero.
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26. Because flies without vertical lobes represent less than 5% of the *ala* population, on average only three to four such flies were present in each conditioning experiment. Calculating a PI for each experiment with so few individuals was meaningless, and therefore *t*- test analyses were not applied here. A global PI was calculated for each category of *ala* flies by adding flies from repeated experiments. This global score represents a mean of individuals' behavior. Statistical analyses were performed with a nonparametric χ^2 test. The reference theoretical value used for χ^2 analysis of *ala* flies with missing lobes was the distribution of *ala* flies with all lobes coming from the same experiments.
27. χ^2 analysis predicts that a population of more than 7000 *ala* flies would have to be run through the massed conditioning protocol and their brain analyzed in order to validate statistically the observed difference.

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SNARE Function Analyzed in Synaptobrevin/VAMP Knockout Mice

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SNAREs (soluble NSF-attachment protein receptors) are generally acknowledged as central components of membrane fusion reactions, but their precise function has remained enigmatic. Competing hypotheses suggest roles for SNAREs in mediating the specificity of fusion, catalyzing fusion, or actually executing fusion. We generated knockout mice lacking synaptobrevin/VAMP 2, the vesicular SNARE protein responsible for synaptic vesicle fusion in forebrain synapses, to make use of the exquisite temporal resolution of electrophysiology in measuring fusion. In the absence of synaptobrevin 2, spontaneous synaptic vesicle fusion and fusion induced by hypertonic sucrose were decreased ~10-fold, but fast Ca^{2+}-triggered fusion was decreased more than 100-fold. Thus, synaptobrevin 2 may function in catalyzing fusion reactions and stabilizing fusion intermediates but is not absolutely required for synaptic fusion.

Intracellular fusion reactions are generally thought to be mediated by SNAREs, a large family of membrane proteins characterized by a common sequence called the SNARE motif (*1–6*). During fusion, SNAREs from opposing membranes form core complexes through their SNARE motifs. Different fusion reactions involve distinct sets of SNAREs, although some SNAREs function in multiple reactions. SNAREs have probably been studied in greatest detail at the synapse where the synaptic vesicle SNARE synaptobrevin (also called VAMP) interacts with the plasma membrane SNAREs syntaxin 1 and SNAP-25. Synaptobrevin is a minimal SNARE that consists only of a short NH_2-terminal sequence, a SNARE motif, and a COOH-terminal transmembrane region. Syntaxin 1, in contrast, contains an NH_2-terminal three-helical domain that interacts with multiple other proteins in addition to a SNARE motif and a membrane anchor, and SNAP-25 includes two SNARE motifs besides a membrane anchor (*6*). In spite of substantial progress in the identification of SNAREs as essential components of membrane fusion reactions, their precise role in fusion has remained enigmatic. Three principal hypotheses have been proposed. First, the original SNARE hypothesis posited that SNAREs determine the specificity of fusion reactions (*7*), and recent in vitro fusion reactions have provided support for this idea (*8*). Second, experiments with yeast vacuoles indicated a role for SNARE complexes preceding the actual fusion reaction, suggesting that complex formation catalyzes the subsequent fusion reaction but does not actually execute it (*9*). Finally, it has been proposed that core

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complex formation is identical with fusion and that the induced proximity of the membranes forces them into fusion (*10*). According to this idea, interacting SNAREs represent the "minimal fusion apparatus" that is both necessary and sufficient for fusion (*11*).

At present, the prevailing view favors the hypothesis that SNAREs execute fusion, but it has been difficult to distinguish among the three hypotheses experimentally. Reconstitution experiments provided evidence for SNAREs as minimal fusion proteins (*11*); however, this in vitro reaction only distantly resembles the physiological protein requirements, kinetics, and Ca^{2+} regulation of fusion. Furthermore, many proteins induce fusion of lipid vesicles; for example, NSF, an adenosine triphosphatase that disassembles SNARE complexes, is a very effective fusion factor (*12*). Fusion assays with purified organelles and permeabilized cells furnished valuable evidence for a role of SNAREs in fusion but were also unable to pinpoint their exact action. In permeabilized PC12 cells, there is not a good correlation between the ability of SNAP-25 mutants to form SNARE complexes and to mediate fusion (*13*), and in Golgi membranes, mutant NSF catalyzes fusion even when unable to dissociate SNARE complexes (*14*). Moreover, recent data have shown that proteolipid channels may execute membrane fusion in yeast vacuoles (*15*) and that deletion of the synaptobrevin homologs snc1/2p at the plasma membrane can be compensated by changes in phospholipid metabolism (*16*). Finally, studies with tetanus and botulinum toxins provided great insight into SNARE function in fusion but also raised important questions. For example, is the residual fusion observed after toxin treatment (*17*, *18*) due to remaining "protected" SNAREs, or does it reflect SNARE-less fusion? A limitation of many previous studies was the low temporal resolution of the assays used, the availability of only a single readout for fusion, and the fact that fusion could usually be induced only by a single stimulus such as Ca^{2+}. To circumvent these problems, we generated mice that lack synaptobrevin 2 and allow a genetic analysis of a SNARE protein in vertebrates using the exquisite temporal resolution and versatility of physiological methods.

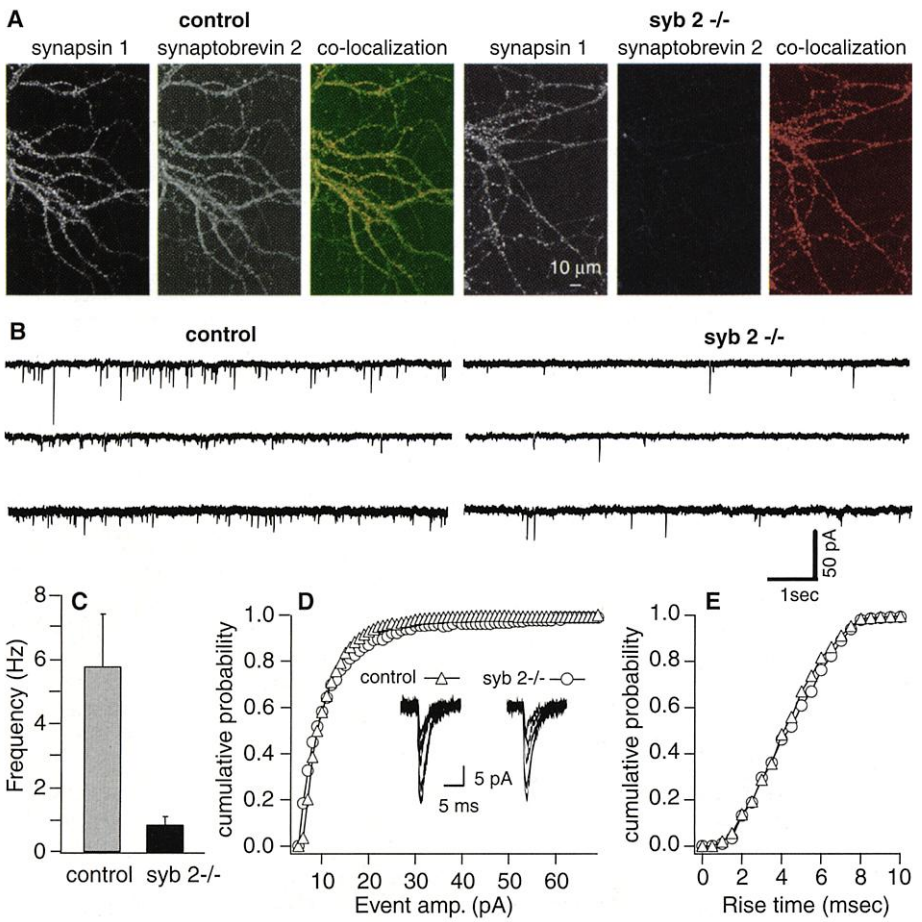

Fig. 1. Spontaneous synaptic responses in cultured hippocampal neurons from mutant and control mice. (**A**) Immunofluorescence staining of cultured neurons. High-density hippocampal cultures from control and synaptobrevin 2 knockout (syb 2−/−) mice were labeled with antibodies to the indicated proteins and viewed in a confocal microscope. (**B**) Representative traces of spontaneous synaptic events in control and synaptobrevin knockout neurons recorded in 1 µM tetrodotoxin. (**C**) Frequency of spontaneous synaptic events. Events in synaptobrevin knockouts (n = 13) had a reduced frequency compared with control neurons (n = 7). (**D** and **E**) Cumulative histograms of current amplitude (D) and rise times (E) from individual synaptic events from mutant or control cultures. The inset in (D) shows exemplary events [n = 6 for wild type (2135 events), n = 5 for syb −/− (274 events)].

We produced mutant mice in which expression of synaptobrevin 2 was abolished by homologous recombination (*19*). Heterozygous mutant mice suffered no apparent morbidity or premature mortality, but homozygous mutant mice died immediately after birth. Newborn knockout mice exhibited a striking body shape, with a rounded appearance and a shoulder hump that is probably caused by excess brown fat in the upper back, but no developmental changes (*19*). Because synaptobrevin 2 is known to function in endocrine and fat cells (*20*–*23*), the altered body shape of the mutant mice could be due to impaired regulated exocytosis in these cells. Analysis of brain sections from synaptobrevin knockout and wild-type control mice did not detect abnormalities (*19*), especially no neurodegeneration similar to that of munc18-1 knockout mice (*24*). Furthermore, no changes were observed in the expression of a series of synaptic proteins (*25*). To evaluate the possibility of redundancy, we examined the levels of synaptobrevin 1/VAMP1 and cellubrevin, the only known SNAREs that are closely related to synaptobrevin 2. Consistent with previous studies (*26*, *27*), even extended exposures of immunoblots from control and knockout mice did not show a signal for synaptobrevin 1 or cellubrevin in forebrain (*19*). Thus, there are no closely related SNAREs in the rostral brain regions of the knockout mice that could substitute for the synaptobrevin 2 deficiency.

To analyze synaptic transmission, we used high-density cultures of hippocampal neurons prepared from embryos (*28*, *29*). All analyses were performed simultaneously on knockout and control cultures derived from littermates to avoid culture-specific artifacts. As viewed by immunocytochemistry, mutant and control neurons formed a similar dense meshwork of synapses (Fig. 1A). We first examined synapse function by measuring spontaneous miniature excitatory currents ("minis") (*29*). Unequivocal minis were recorded in synaptobrevin-deficient neurons (Fig. 1B), but with a reduced frequency (~15% of control neurons; Fig. 1C). Thus, a synaptic release apparatus, operating at a lower efficiency, was present in the synaptobrevin knockouts, as also seen in *Drosophila* and *Caenorhabditis elegans* synaptobrevin mutants (*30*, *31*). Synaptobrevin may be an essential determinant of fusion pore opening rates (*32*) or of the assembly of postsynaptic

specializations (*33*). However, amplitudes and rise times of the minis in synaptobrevin-deficient neurons were indistinguishable from controls (Fig. 1, D and E), suggesting that the synaptobrevin knockout did not cause a change in the rate of fusion pore opening or the concentration of postsynaptic receptors.

We next tested if neurotransmitter release could be evoked in the mutant neurons by action potentials that trigger Ca^{2+} influx (*29*). In recordings from single control neurons stimulated at 1 Hz, we observed reliable synaptic responses with large amplitudes (Fig. 2A). Synaptic amplitudes were relatively constant for each neuron but differed between neurons, presumably because they reflect a variable number of simultaneous synaptic inputs. Stimulation of mutant neurons, by contrast, elicited only rare synaptic events with small amplitudes corresponding to minis (Fig. 2A). Even at high frequencies (10 to 30 Hz), only erratic events occurred that were not completely synchronous with the stimulation (*29*).

The discrepancy between the persistence of spontaneous release (Fig. 1, B and C) in the absence of evoked release (Fig. 2A) is puzzling, suggesting either that the two measurements monitor separate secretory pathways or that synaptic secretion becomes relatively insensitive to Ca^{2+} in the knockout. To differentiate between these two possibilities, we tested the response of mutant synapses to hypertonic sucrose, which acts on the same vesicles as Ca^{2+} but stimulates the entire pool of readily releasable vesicles (*34*). Twenty-four out of 29 cells tested exhibited a measurable synaptic response to sucrose, with an average amplitude that was 10-fold reduced compared with controls (Fig. 2B). Thus, although the sucrose response was decreased in the synaptobrevin knockouts, it was much less impaired than the nearly immeasurable response to action potentials. The relative functionality of fusion in the synaptobrevin knockout is surprising considering the widely held belief that SNAREs are essential for executing fusion. To ensure that the cultured neurons did not develop a compensatory change in the expression of proteins that are closely related to synaptobrevin 2 (and could potentially substitute), we analyzed the cultures by immunoblotting. However, no synaptobrevin 1 or cellubrevin was detected in the cultures from control or knockout mice, and no substantial change was found in the levels of other synaptic proteins tested (Fig. 2C).

The electrophysiological recordings indicated that without synaptobrevin 2, synaptic fusion is still operational but cannot be efficiently stimulated by Ca^{2+}. To obtain independent evidence for this conclusion, we examined the dynamics of synaptic vesicles in individual synaptic boutons labeled with the fluorescent dyes FM1-43 or FM2-10. When FM dyes are taken up into synaptic vesicle membranes during endocytosis, they become fluorescent; upon exocytosis, the dyes are released, which results in fluorescence destaining that can be monitored (*35*, *36*). Both FM1-43 and 2-10 were tested to avoid dye-specific differences in fluorescence detection. Upon application of a strongly depolarizing stimulus (47 mM K^+ for 90 s), mutant neurons could be stained with FM1-43 or FM2-10 to a level that was about three times lower in intensity than in control neurons (*37*). However, three rounds of K^+ stimulation achieved a degree of fluorescent labeling in mutant neurons that approached the fluorescence of control boutons, although with a broader distribution (Fig. 3A). Not all synapses in the synaptobrevin knockout neurons appeared to be labeled above detection threshold because the apparent density of fluorescent spots was lower than the density of synapses observed by immunocytochemistry, suggesting that only a subset of synapses was stimulated during loading.

We applied a strongly depolarizing stimulus (90 mM K^+), which triggers Ca^{2+} influx to the labeled neurons to monitor the release of FM1-43 or FM2-10 as a measure of synaptic vesicle fusion. Control synapses exhib-

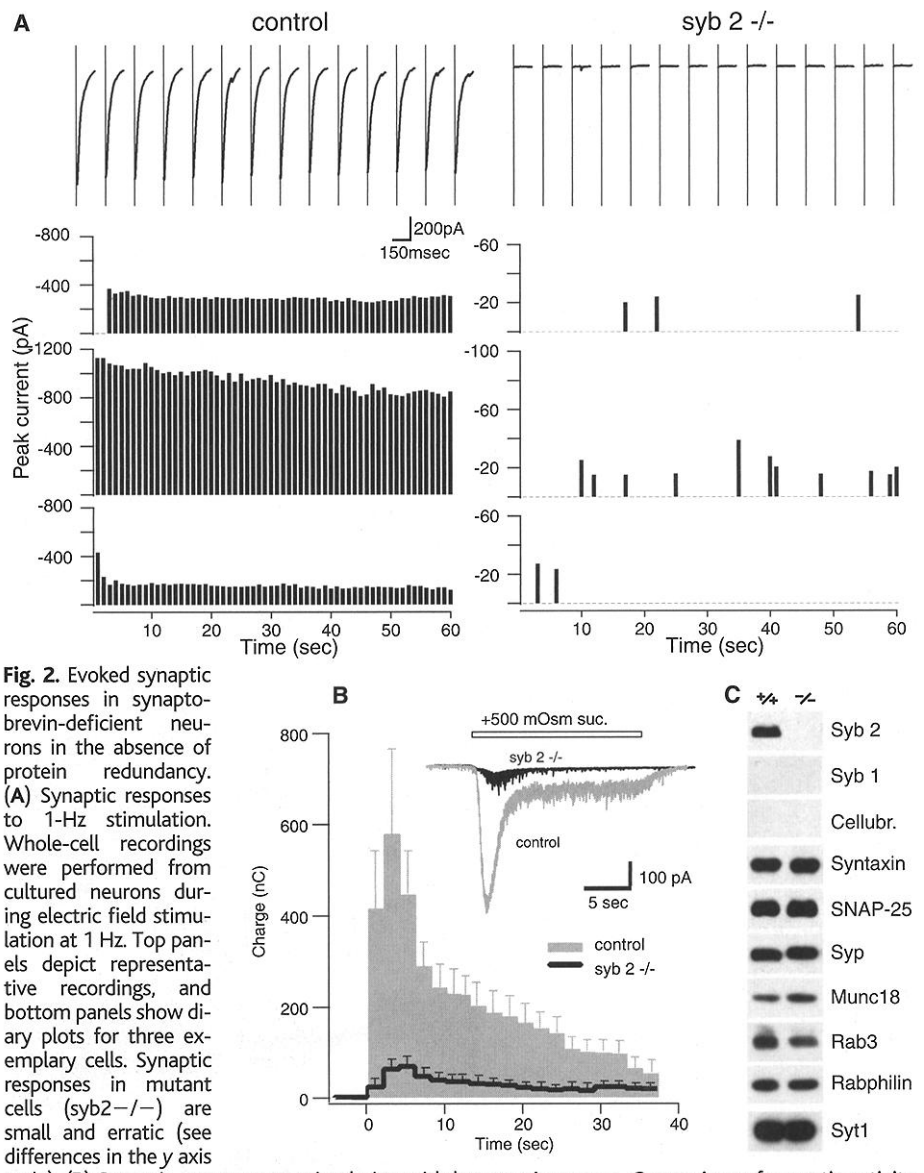

Fig. 2. Evoked synaptic responses in synaptobrevin-deficient neurons in the absence of protein redundancy. (**A**) Synaptic responses to 1-Hz stimulation. Whole-cell recordings were performed from cultured neurons during electric field stimulation at 1 Hz. Top panels depict representative recordings, and bottom panels show diary plots for three exemplary cells. Synaptic responses in mutant cells (syb2−/−) are small and erratic (see differences in the *y* axis scale). (**B**) Synaptic responses to stimulation with hypertonic sucrose. Comparison of synaptic activity induced by application of hypertonic sucrose (0.5 osm) in control (n = 6) and mutant neurons (n = 9). The graph depicts cumulative charge transfer integrated over 2-s intervals during sucrose application. (Inset) Representative traces from a mutant and a control cell depicting the time course and amplitude of synaptic responses to hypertonic sucrose. (**C**) Immunoblots of cultured neurons. Cultures used for electrophysiology were analyzed by immunoblotting for the indicated proteins to document that there are no changes in the expression of potentially redundant synaptobrevin isoforms during the period of culture.

ited a major fast component of destaining and a minor slow component. In contrast, mutant synapses completely lacked the fast component and displayed only a slow fluorescence decline (Fig. 3B). Furthermore, ~25% of labeled boutons were refractory to stimulation. Thus, fast Ca^{2+}-triggered neurotransmitter release was largely absent from the mutant neurons as observed electrophysiologically, whereas the slow phase persisted (which explains the ability of strong stimulation to stain the mutant synapses). To test if the rate of destaining was sensitive to Ca^{2+}, we determined the fluorescence decline after 15 s of stimulation at different extracellular Ca^{2+} concentrations. Changes in Ca^{2+} shifted the rate of destaining of wild-type synapses substantially (Fig. 3C) but had no effect on mutant synapses (Fig. 3D). In these measurements, changes in Ca^{2+} affect vesicle mobilization but not release probability because the strong 90 mM K^+ stimulation already makes the initial release probability maximal (Fig. 3). Thus, the lack of an effect of increasing Ca^{2+}-concentrations on mutant neurons—neither the fraction of boutons that respond nor the extent of destaining was altered—suggests that deletion of synaptobrevin 2 not only blocks fast Ca^{2+}-triggered release but also abolishes Ca^{2+}-dependent increases in the extent of release.

The optical results, similar to the electrophysiological recordings, suggest that fast Ca^{2+}-triggered release is impaired, but a slow release phase is maintained in the synaptobrevin knockouts. To determine if the readily releasable pool stimulated by hypertonic sucrose is relatively less impaired as indicated electrophysiologically, we directly compared the two stimulation paradigms in the same experiment. We stimulated neurons either first with hypertonic sucrose for 30 s followed by a 60-s recovery period and extended exposure to 90 mM K^+ (Fig. 4A) or first with 90 mM K^+ for 60 s followed by a 5- or 120-s recovery period and exposure to hypertonic sucrose (Fig. 4, B and C).

Initial application of hypertonic sucrose resulted in rapid destaining of control synapses (mean fluorescence decrease ~25%). Subsequent challenges of the synapses with 90 mM K^+, a strongly depolarizing agent, caused further rapid and extensive fluorescence loss (Fig. 4A). In mutant synapses, application of hypertonic sucrose also induced rapid destaining, although with a lower average fluorescence decrease (~12%; Fig. 4A). The rapid onset of sucrose-induced synaptic responses was in striking contrast to their slow response to Ca^{2+} influx triggered by high K^+. However, ~30% of mutant synapses were refractory to sucrose (Fig. 4A) as observed for stimulation with high K^+ (Fig. 3D). K^+ stimulation after hypertonic sucrose elicited only a weak monophasic response (Fig. 4A). Thus, similar to the electrophysiology, hypertonic sucrose is disproportionately more effective than high K^+ in triggering fusion monitored with FM dyes.

To ensure that this conclusion did not depend on the sequence in which the stimulations were performed—for example, the possibility that the sucrose stimulus exhausts the readily releasable pool so that subsequent K^+ stimulation finds no remaining releasable vesicles—we compared the two stimulation methods in reverse order. We first applied 90 mM K^+ for 60 s, then allowed recovery for 5 or 120 s, and finally stimulated with hypertonic sucrose. In control neurons, the relative amount of destaining induced by hypertonic sucrose is small compared with destaining elicited by the prior strong K^+ stimulus, independent of the length of the recovery period between the two stimuli (Fig. 4B). By contrast, the proportion of release triggered by hypertonic sucrose was much larger in the synaptobrevin mutants even when applied after a strong K^+ stimulus (Fig. 4C). Sucrose-induced destaining was relatively larger after 5-s recovery than after 120-s recovery, presumably because undocking and mixing of dye-loaded vesicles occur during the recovery period. These result validate the observation that in a majority of mutant synapses, hypertonic sucrose is more effective in triggering fusion than Ca^{2+}-dependent stimulation.

In the present study, we show that synapses deficient in synaptobrevin 2, the only SNARE protein of its class that is present in the synapses analyzed, exhibit a ~10-fold decrease in spontaneous synaptic vesicle fu-

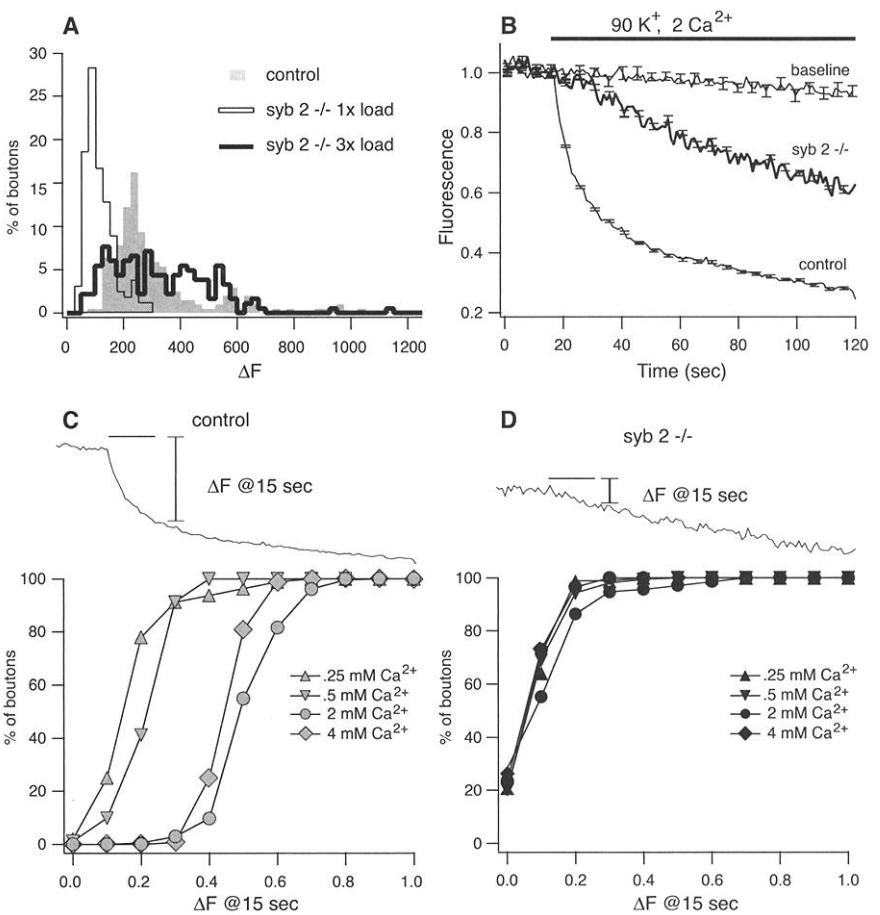

Fig. 3. Stimulated vesicle recycling in synaptobrevin-deficient synapses detected by uptake and release of fluorescent FM1-43 and FM2-10. (**A**) Fluorescence loading of synaptic vesicles with FM2-10 in control and mutant synapses. Histograms display the amount of FM2-10 fluorescence staining of control synapses (gray silhouette; n = 204) and mutant synapses (thin line; n = 155 boutons) induced by a single application of 47 mM K^+ for 90 s and the amount of fluorescence in mutant synapses after three applications of the same stimulus (dark line, n = 182 boutons). (**B**) Fluorescence destaining of mutant and control synapses in response to 90 mM K^+ stimulation. Mutant synapses loaded with FM1-43 (n = 95) or FM2-10 (n = 152) and control synapses loaded with FM2-10 (n = 204) by a single round of K^+ depolarization were stimulated with 90 mM K^+ or control solution (baseline). (**C** and **D**) Cumulative histograms depicting the extent of fluorescence loss within 15 s of 90 mM K^+ application at different extracellular Ca^{2+} concentrations. In control synapses, destaining kinetics became progressively faster with increasing $[Ca^{2+}]_e$ up to 4 mM Ca^{2+}, consistent with previous observations (*36*). In mutant synapses, increasing $[Ca^{2+}]_e$ from 0.25 mM to 4 mM did not alter the kinetics of destaining (n = 100 to 150 boutons per $[Ca^{2+}]_e$ for each genotype).

sion and a similar decrease in sucrose-triggered fusion, but a >100-fold decrease in Ca^{2+}-induced fusion. Because hypertonic sucrose and Ca^{2+} stimulate the fusion of the same vesicles, although by different mechanisms (*34*), this finding suggests that the SNARE protein synaptobrevin 2 is not required for synaptic fusion as such but is essential for a normal rate of fusion upon stimulation. These results can be explained by at least three hypotheses:

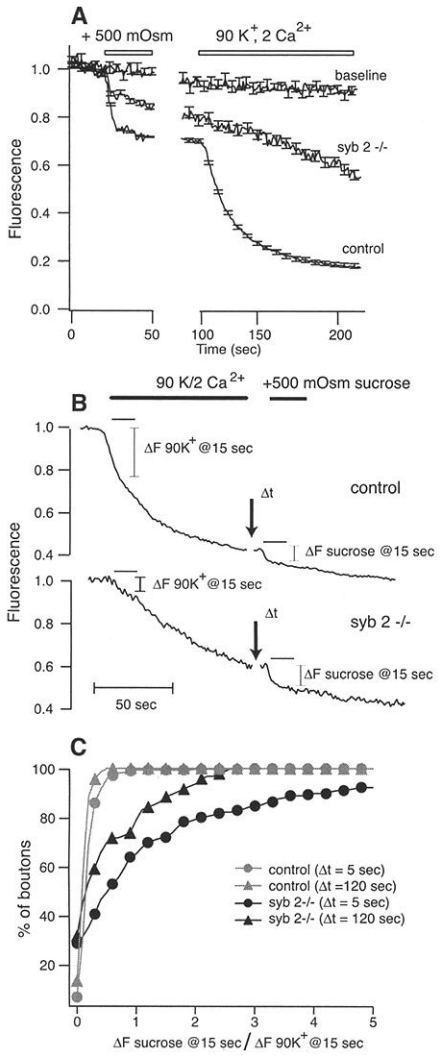

Fig. 4. Comparison of synaptic fusion triggered by high K^+ and hypertonic sucrose. (**A**) Stimulation of fusion by hypertonic sucrose (0.5 M sucrose) followed by K^+ depolarization (90 mM K^+). A 60-s recovery period was interposed between the two stimuli. Hypertonic sucrose causes rapid FM2-10 destaining also of synaptobrevin-deficient synapses, whereas K^+ depolarization does not (n = 331 boutons for mutant and n = 214 boutons for control synapses). (**B** and **C**) Stimulation of fusion by K^+ depolarization (90 mM K^+) followed by hypertonic sucrose (0.5 M sucrose). Control and mutant synapses loaded with FM2-10 were exposed to 90 mM K^+ for 90 s, followed by a 5- or 120-s recovery period and a 30-s pulse of hypertonic sucrose. (B) depicts optical traces that were used to calculate the ratio of fluorescence loss at 15 s, and (C) summarizes the ratios as histograms. Sucrose responses obtained with a 5-s recovery period were larger than responses with a 120-s recovery period, presumably because dye-loaded vesicles and unlabeled vesicles mix during recovery (control: n = 303 for Δt = 5 s, n = 204 for Δt = 2 min; syb2−/−: n = 235 for Δt = 5 s, n = 95 for Δt = 2 min).

The primary function of SNAREs in fusion is catalytic to increase the rate of fusion reactions like an enzyme by stabilizing transition states. This hypothesis proposes that synaptic vesicles go through progressive stages in preparation for exocytosis, from docked over sucrose-responsive to Ca^{2+}-responsive vesicles. If stabilization of these stages required SNARE complexes, this would explain why the readily releasable pool is impaired in the synaptobrevin knockout, but less so than the Ca^{2+}-responsive pool. According to this hypothesis, fusion does not actually require SNARE complexes, but SNAREs are essential because they are rate-limiting for fusion. This hypothesis provides a good agreement of the synapse data with yeast studies in which SNARE complexes have been shown to assemble before the actual fusion reaction (*9*) and deletion of the synaptobrevin homologs snc1/2p was overcome by changes in phospholipid metabolism (*16*).

Independent of which hypothesis will prove correct, our data assign to synaptobrevin 2 a preferential role in the Ca^{2+} triggering of fusion. However, different from the synaptotagmin or complexin knockouts where only a deficiency in Ca^{2+}-triggered but not in sucrose-mediated release was found (*44*, *45*), synaptobrevin is also essential for a large component of the readily releasable pool. It is interesting that the synaptic core complex has potential Ca^{2+}-binding sites on its surface (*42*) and that inhibition of core complex formation with antibodies selectively inhibits fast Ca^{2+}-triggered fusion in chromaffin cells (*46*). Although the latter experiments were interpreted in a model with different types of SNARE complexes, a more straightforward explanation in view of our results would be that full SNARE complexes are not required for fusion as such but catalyze formation of transition states.

2) Other, noncognate SNAREs that normally do not function in synaptic vesicle fusion could potentially substitute for synaptobrevin 2 in the mutant neurons. This hypothesis agrees well with the fact that tight core complexes are formed by noncognate SNAREs (which do not normally function in the same cellular pathways) (*38*, *39*). However, it is difficult to envision how noncognate SNAREs could be recruited into the synaptic vesicle pathway (*40*), and this hypothesis would imply that SNAREs perform two separate functions in membrane fusion: (i) an executive function in which different SNAREs are promiscuous and (ii) a regulatory function for which a particular SNARE is specific. Similar reservations apply to the possibility that functional t-SNARE complexes are assembled by syntaxin 1 and SNAP-25 on synaptic vesicle and plasma membranes (*41*) because such t-SNARE complexes differ structurally from regular SNARE complexes (*42*, *43*). Although these hypotheses seem thus unlikely, we cannot completely rule them out.

1) Although we did not detect immunoreactivity for synaptobrevin 1 or cellubrevin in our cultures, it is possible that these proteins are present below the detection limit and mediate the observed residual fusion. However, two findings argue against this possibility. First, at least 10% of sucrose-evoked release is intact in the mutant neurons, but the sensitivity of our antibodies would certainly have detected redundant synaptobrevin 1 and cellubrevin at such levels. Second, if low levels of these synaptobrevin isoforms were present and executed the residual fusion, sucrose- and Ca^{2+}-dependent release should be impaired equally instead of the differential effect we observe.

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25. Forebrain proteins from mutant and wild-type mice were studied by immunoblotting; antibody signals were quantified on blots reacted with test and control antibodies (to annexin 6 or guanine nucleotide disassociation inhibitor) as internal standards and

with ^{125}I-labeled secondary antibodies. Levels were measured in a phosphoimager and normalized for the internal standards to control for variations in transfer or secondary antibodies. No synaptobrevin 1 or cellubrevin was detected in forebrain, and no significant changes ($P < 0.01$) were found for SNAP-25, syntaxin 1, synaptophysin, NSF, Munc18-1, rab3A, synaptotagmin 1, Vap-33, CASK, Mint1, rabphilin, rabaptin, synapsins 1 and 2, and RIMs 1 and 2 [see supplementary materials (*47*)].

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29. Neurons from embryonic day 18 hippocampus were cultured as described in the supplementary materials (*47*). Synaptic responses were monitored with whole-cell patch clamp recordings with an Axopatch 200B amplifier and Clampex 8.0 software (Axon Instruments, Union City CA) filtered at 1 kHz and sampled at 200 μs. Recordings were made in modified Tyrode solution [composition: 150 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes, and 2 $CaCl_2$ (pH 7.4, 310 mosm)], with an internal pipette solution containing 115 mM Cs-$MeSO_3$, 10 mM CsCl, 5 mM NaCl, 10 mM Hepes, 0.6 mM EGTA, 20 mM tetraethylammonium -Cl, 4 mM Mg–adenosine triphosphate, 0.3 mM Na_2–guanosine triphosphate, and 10 mM QX-314 (pH 7.35, 300 mosm). Spontaneous release was recorded with 1 μM tetrodotoxin in the medium and without QX-314 in the pipette. Field stimulations (1-ms pulses of 30 mA) were applied at various frequencies through parallel platinum electrodes immersed into the perfusion chamber. Hypertonic solution (0.5 M) was applied in Tyrode's medium.
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37. Synaptic boutons were loaded with FM2-10 (400 μM) or FM1-43 (8 μM) (Molecular Probes, Eugene, OR) in Tyrode's solution (*29*) containing 47 mM K^+ (to stimulate uptake) and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione and 50 μM AP-5 (to prevent recurrent activity) and washed for 10 min with the same solution with regular K^+ and without Ca^{2+} (to minimize spontaneous dye loss) (*36*). Synaptic vesicle fusion was induced by field stimulations (*29*) or by local gravity perfusion of hypertonic or hyperkalemic solutions onto the field of interest (1 to 2 ml/min). Adjustment of hyperosmotic solution flow rate was critical to prevent alterations in fluid levels and fluorescence values during rapid solution exchanges. Fluorescence values were not substantially distorted by cell shrinkage during sucrose application. In all experiments, we analyzed isolated boutons (~1 μm^2) and avoided apparent synaptic clusters. Images were acquired with a cooled-intensified digital charge-coupled device (CCD) camera (Roper Scientific, Trenton, NJ) during illumination (1 Hz, 15 ms) at 480 ± 20 nm (505 DCLP, 535 ± 25 BP) through an optical switch (Sutter Instruments, Novato, CA) and processed with Metafluor Software (Universal Imaging, Downingtown PA).
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47. Supplementary material is available on *Science* Online at www.sciencemag.org/cgi/content/full/294/5544/1117/DC1
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