

# SNAREs in native plasma membranes are active and readily form core complexes with endogenous and exogenous SNAREs

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**D**uring neuronal exocytosis, the vesicle-bound soluble NSF attachment protein (SNAP) receptor (SNARE) synaptobrevin 2 forms complexes with the plasma membrane-bound SNAREs syntaxin 1A and SNAP25 to initiate the fusion reaction. However, it is not known whether in the native membrane SNAREs are constitutively active or whether they are unable to enter SNARE complexes unless activated before membrane fusion. Here we used binding of labeled recombinant SNAREs to inside-out carrier supported plasma membrane sheets of PC12 cells to probe for the activity of endogenous SNAREs. Binding was

specific, saturable, and depended on the presence of membrane-resident SNARE partners. Our data show that virtually all of the endogenous syntaxin 1 and SNAP-25 are highly reactive and readily form SNARE complexes with exogenously added SNAREs. Furthermore, complexes between endogenous SNAREs were not detectable when the membranes are freshly prepared, but they slowly form upon prolonged incubation *in vitro*. We conclude that the activity of membrane-resident SNAREs is not downregulated by control proteins but is constitutively active even if not engaged in fusion events.

## Introduction

Soluble NSF attachment protein (SNAP)\* receptor (SNARE) proteins comprise a superfamily of small, mostly membrane-bound, proteins that are essential for membrane fusion (Jahn and Südhof, 1999; Chen and Scheller, 2001). Characteristic of all SNAREs are the SNARE motifs, homologous stretches of ~60 amino acids that are adjacent to the membrane anchor domains. SNARE motifs are unstructured as monomers. However, when appropriate sets of SNAREs are mixed, their SNARE motifs assemble spontaneously into tight complexes of extraordinary stability, also referred to as core complexes. Disassembly of core complexes is mediated by the action of the chaperone-like ATPase NSF in conjunction with cofactors (Söllner et al., 1993). Crystallographic studies have shown that core complexes consist of elongated four helix bundles in which the helices are connected by 16 layers of

highly conserved amino acid side chains. The membrane anchor domains are extending at the COOH-terminal end of the bundle. Each helix is contributed by a different SNARE motif that occupies a unique position in the complex (Sutton et al., 1998; Antonin et al., 2002).

Membrane fusion requires the formation of core complexes (Chen and Scheller, 2001; Bruns and Jahn, 2002). When membrane contact is established, appropriate sets of SNAREs assemble into trans-complexes that connect the membranes. It is presently believed that assembly proceeds from the distal NH<sub>2</sub>-terminal ends of the SNARE motifs towards the COOH-terminal membrane anchors, thus pulling the membranes closely together and overcoming the energy barrier for fusion (Hanson et al., 1997). Proteoliposomes carrying appropriate sets of SNAREs spontaneously fuse (Weber et al., 1998). Furthermore, interference with the assembly of trans complexes blocks fusion (Xu et al., 1999; Chen et al., 2001) supporting the view that SNARE assembly suffices to catalyze membrane merger. After fusion, all transmembrane domains are aligned in parallel and reside in the same membrane (cis-complex). Cis-complexes are inactive and need to be dissociated by NSF in order to regenerate active and fusion-competent SNARE proteins.

Considering that free SNARE motifs spontaneously form SNARE complexes *in vitro*, the question that arises is how

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\*Abbreviations used in this paper: BoNT, botulinum neurotoxin; FRET, fluorescence resonance energy transfer; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; TeNT, tetanus neurotoxin; TMA-DPH, 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene.

Key words: exocytosis; membrane fusion; PC12 cells; *in vitro*; SNARE reactivity

their activity is controlled during membrane traffic. Several lines of evidence were used to suggest that membrane-resident SNAREs are normally inactive and require activation before fusion (Pfeffer, 1999; Wickner and Haas, 2000). For instance, when the fusion of endosomes, yeast vacuoles, or of exocytotic secretory vesicles is assayed *in vitro*, the action of ATP-NSF is needed before fusion can occur. Therefore, NSF and its cofactors are considered to belong to the priming factors that are needed to establish fusion competence (Avery et al., 1999). Because NSF also acts upon partially assembled complexes and individual SNAREs (Hanson et al., 1995; Hayashi et al., 1995), its role may not be confined to the disassembly of core complexes, but rather include a general conformational activation of SNARE proteins.

In addition, the activity of SNAREs may be regulated by other proteins. A large number of proteins have been shown to bind specifically to certain SNAREs, and some of them may control the ability of the SNAREs to enter core complexes. For instance, several dozen binding proteins are reported for the SNAREs functioning in neuronal exocytosis that include the synaptic vesicle protein synaptobrevin (also referred to as vesicle-associated membrane protein) and the plasma membrane proteins SNAP-25 and syntaxin 1. Binding of the protein Munc-18 to syntaxin 1 *in vitro* stabilizes the interaction of the NH<sub>2</sub>-terminal domain of syntaxin 1 with the SNARE-motif, preventing syntaxin from binding to its SNARE partners (Yang et al., 2000). Because several other SNAREs possess NH<sub>2</sub>-terminal domains that fold back onto the SNARE motif (Munson et al., 2000; Tochio et al., 2001), regulation of this intramolecular interaction by other proteins may be a general mechanism for controlling SNARE activity. Another example is the binding between synaptobrevin and the vesicle protein synaptophysin which prevents interactions with the SNARE-partners syntaxin and SNAP-25 (Edelmann et al., 1995; Washbourne et al., 1995). Furthermore, GTPases of the rab/ypt protein families have been invoked in the control of SNAREs (Lupashin and Waters, 1997; Wickner and Haas, 2000) although the mechanism is still unclear.

Although these and many additional data suggest that the SNAREs are tightly regulated, there is only scant information about the size and activity status of SNARE pools in intact cells and membranes. Using several independent experimental approaches, it was shown for the synaptic SNAREs that cis-complexes do exist in intact membranes (Otto et al., 1997), and that their concentration appears to be up-regulated during exocytotic activity (Lonart and Sudhof, 2000; Sanyal et al., 2001). Addition of recombinant SNAREs to cell-free fusion reactions was shown to compete with, or to substitute for, cleaved native SNAREs in membrane fusion, suggesting that the pool of SNAREs involved in exocytosis is at least transiently accessible to external probes (Chen and Scheller, 2001). However, it is not known whether membrane-resident SNAREs not engaged in ongoing fusion events are active with respect to their ability to form core complexes, or whether they are downregulated by other factors.

In the present study, we have used recombinant and fluorescently labeled SNAREs as probes to investigate to which extent the SNARE motifs of membrane-resident SNAREs are capable of binding other SNAREs. As paradigms, we used the SNAREs functioning in neuronal exocytosis. We

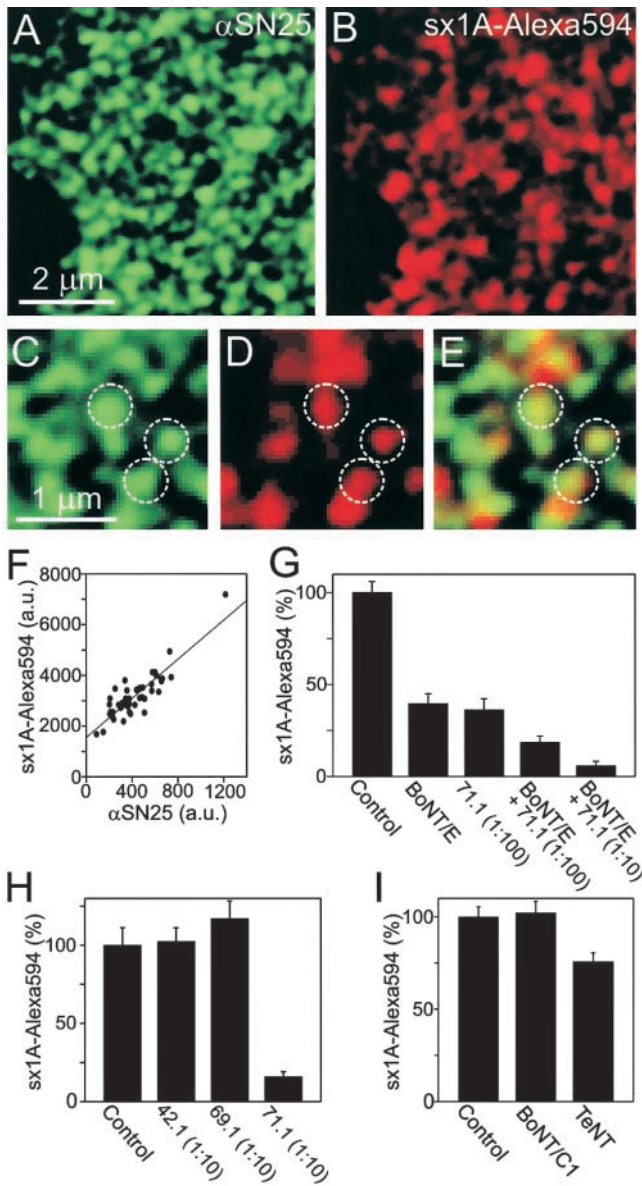
took advantage of a recently developed procedure for preparing inside-out sheets of carrier supported plasma membranes from PC-12 cells. These sheets retain their capacity for exocytosis, and the membrane-resident SNAREs are freely accessible to external probes. Surprisingly, we found that recombinant SNAREs readily bind to the membranes and that binding is due to complex formation with endogenous SNAREs. Virtually all of the endogenous SNAREs are active in freshly prepared membrane sheets, and they are not complexed with other SNAREs. However, upon extended incubation they spontaneously assemble into inactive cis-complexes, thus losing their ability to bind exogenous SNAREs.

## Results

### Exogenous syntaxin 1A binds to endogenous SNAP-25 in inside-out plasma membrane sheets

We have previously shown (Lang et al., 2001) that both syntaxin 1 and SNAP-25 form cholesterol-dependent clusters in the plasma membrane that only partially overlap with each other, thus allowing for differentiating by fluorescence microscopy syntaxin and SNAP-25 clusters. *In vitro*, syntaxin and SNAP-25 form stable binary 2:1 complexes that are structurally similar to, but less stable than, core complexes. To examine if membrane-resident SNAP-25 is capable of forming complexes with exogenously added syntaxin, we incubated inside-out, glass-adhered plasma membrane sheets prepared from PC12 cells (Avery et al., 2000) with recombinant syntaxin. The syntaxin variant used in this and all subsequent assays lacked its transmembrane domain and was labeled with the fluorophore Alexa594 at a cysteine introduced in position 171. As shown in Fig. 1 (A–E), we observed binding of syntaxin that was not uniform but concentrated in spots, many of which were immunoreactive for SNAP-25 (Fig. 1, A–E). To obtain an estimate of the relationship between syntaxin binding and the density of SNAP-25, we quantitated the fluorescence signals using two different methods. First, we took random pictures and selected areas of approximately 5–10  $\mu\text{m}^2$  followed by quantitation of fluorescence intensities in both channels (see Materials and methods). A linear correlation between endogenously available SNAP-25, as judged by the immunostaining intensity for SNAP-25 (green channel), and syntaxin 1A-Alexa594 fluorescence (red channel), was observed (Fig. 1 F). Second, we determined the degree of colocalization between bound syntaxin and SNAP-25 at the level of individual fluorescent spots (Fig. 1; Materials and methods). The background-corrected degree of colocalization was  $45 \pm 5\%$ .

The moderate degree of colocalization can be explained in two ways. First, binding of syntaxin may interfere with immunostaining of SNAP-25, which is likely because most known immunoreactive epitopes are within the SNARE-motifs (Xu et al., 1999). Second, it cannot be excluded that there are other binding sites for syntaxin, an interpretation also suggested by the fact that there is still significant syntaxin binding in absence of detectable SNAP-25 immunoreactivity (Fig. 1 F). To differentiate between these possibilities, we incubated the membrane sheets with the light chain of botulinum neurotoxin (BoNT) and the SNAP-25-specific monoclonal antibody Cl 71.1, both reported to inter-



**Figure 1. Syntaxin 1A binds to endogenous SNAP-25 on inside-out sheets of plasma membrane derived from PC12 cells.** (A and B) Membrane sheets were reacted for 50 min at 37°C with syntaxin 1A(1–262)–Alexa594 (red channel) and then washed, fixed, and immunostained for SNAP-25 (green channel). (C and D) Magnified views from A and B. (E) Overlap from C and D. Circles indicate identical pixel locations. (F) On membrane sheets such as shown in A and B the staining intensity of random images in the green channel was measured and plotted against the staining intensity in the red channel, showing a linear correlation. (G) Effect of SNAP-25 cleavage by 2  $\mu$ M of BoNT/E and/or CI 71.1 (antibody specific for SNAP-25) on syntaxin binding, measured as above. Dilution of the antibody as indicated. (H) Effect of antibodies against rab3 (CI 42.1), synaptobrevin 2 (CI 69.1), and SNAP-25 (CI 71.1) on syntaxin binding. (I) Binding of syntaxin in the presence of 2  $\mu$ M BoNT/C1 (cleaving syntaxin) and 2  $\mu$ M TeNT (cleaving synaptobrevin).

fere with syntaxin binding to SNAP-25 via independent mechanisms. BoNT/E cleaves SNAP-25 within the COOH-terminal SNARE motif (Binz et al., 1994) reducing its ability to interact with other SNAREs (Hayashi et al., 1994). Antibody CI 71.1 binds to the NH<sub>2</sub>-terminal SNARE motif

and completely abolishes SNARE binding of SNAP-25 in vitro (Xu et al., 1999).

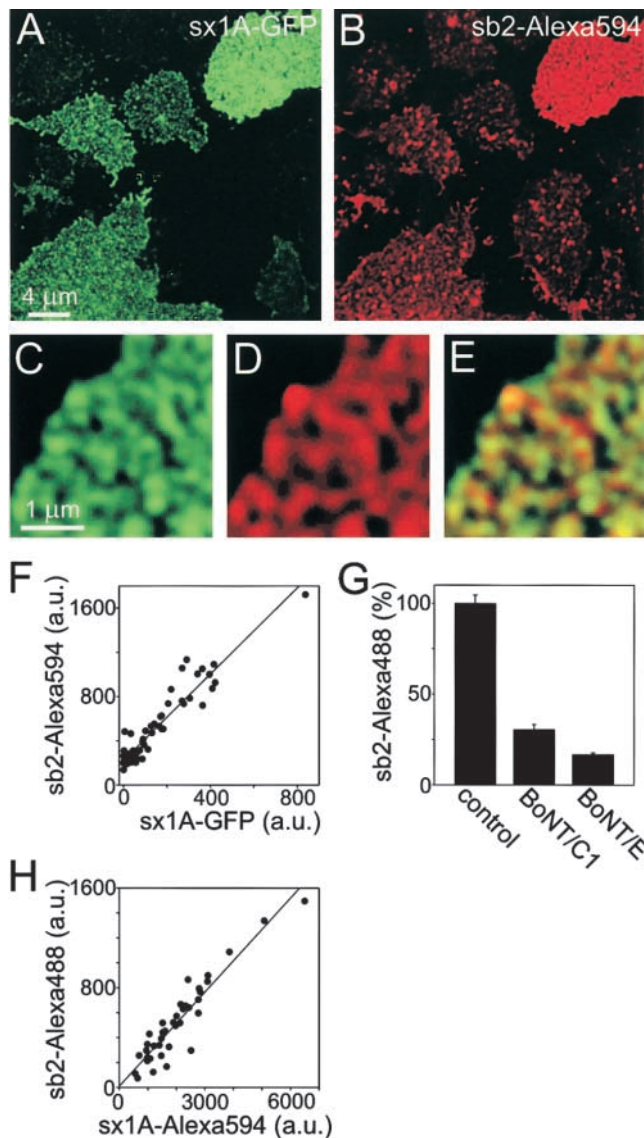
As shown in Fig. 1 G, addition of both BoNT/E and antibody CI 71.1 to the binding reaction strongly interfered with syntaxin binding. Simultaneous incubation with both inhibitors led to a further reduction, and binding was virtually abolished when the concentration of the antibody was increased. In contrast, no inhibition was observed when monoclonal antibodies specific for rab3 or for synaptobrevin were used (Fig. 1 H), although these antibodies effectively labeled their respective antigens in control experiments (unpublished data). These data show that most, if not all, of the syntaxin binding can be accounted for by specific binding to SNAP-25.

No change was observed upon pretreatment with BoNT/C1 which cleaves membrane-bound syntaxin close to the transmembrane domain (Blasi et al., 1993; Schiavo et al., 1995) although endogenous syntaxin was completely removed (see below; see Fig. 4). Thus, it appears that endogenous syntaxin does not interfere with the binding of exogenously added syntaxin. This suggests that endogenous syntaxin does not recruit large pools of SNAP-25 into stable binary complexes. Interestingly, treatment of the membrane sheets with tetanus neurotoxin (TeNT) slightly reduced syntaxin binding (75% of control). Because TeNT specifically cleaves synaptobrevin without affecting any of the other SNAREs (Schiavo et al., 1992), these data suggest that a pool of synaptobrevin in the membrane contributes to the binding of exogenous syntaxin, probably by forming SNARE core complexes that are known to be considerably more stable than binary complexes between SNAP-25 and syntaxin (see below).

### Binding of exogenous synaptobrevin 2 to inside-out plasma membranes depends on both endogenous syntaxin and SNAP-25

We next asked if fluorescently labeled synaptobrevin binds to the membrane sheets. For binding, we used a synaptobrevin 2 variant lacking the transmembrane domain (residue 1–96) that was labeled with Alexa488 or Alexa594 at a cysteine introduced at position 28. Again, binding to membrane clusters was observed. To examine the dependence of synaptobrevin binding on the syntaxin levels in the plasma membrane, membrane sheets were generated from PC12 cells overexpressing syntaxin 1A–GFP, resulting in highly variable expression levels (Fig. 2 A, green channel), with maximal levels exceeding the endogenous levels by 10–15-fold as determined by immunostaining for syntaxin (unpublished data). Bound synaptobrevin colocalized with syntaxin-GFP, with 95  $\pm$  1% of the syntaxin clusters containing bound synaptobrevin, and 91  $\pm$  3% of all synaptobrevin-Alexa594-positive clusters corresponding to a syntaxin-GFP cluster. Furthermore, the amount of bound synaptobrevin correlated with the expression levels of syntaxin-GFP (Fig. 2, A–E and F), resulting in a linear correlation between GFP and Alexa 594 fluorescence, with the intercept on the Y-axis representing binding to the endogenous syntaxin pool.

Previous in vitro studies have shown that synaptobrevin only binds upon core complex formation and does not interact with the free SNARE motifs of either SNAP-25 or syntaxin (Fasshauer et al., 1997b). Therefore, we again added



**Figure 2. Synaptobrevin 2 binds to endogenous syntaxin clusters on inside-out membrane sheets but also requires endogenous SNAP-25 for binding.** (A and B) Membrane sheets were produced from cells expressing syntaxin 1A-GFP (green channel), and then reacted with synaptobrevin 2-Alexa594 (red channel). (C and D) Magnified views from A and B. (E) Overlap from C and D. (F) Plotting green against red fluorescence intensity from membrane sheets as shown in A and B. (G) Synaptobrevin 2 binding depends both on intact syntaxin 1 and SNAP-25, as it is inhibited by membrane treatment with either 2  $\mu$ M BoNT/C1 or BoNT/E. (H) Membrane sheets were reacted with syntaxin 1A-Alexa594 for 60 min, washed two times at room temperature in BSA-KGlu buffer, and then reacted for 50 min with synaptobrevin 2-Alexa488. In this experiment, the measured green fluorescence intensity is an underestimate due to FRET occurring from the green to the red label. Note that in the experiments shown in G and H, nontransfected cells were used.

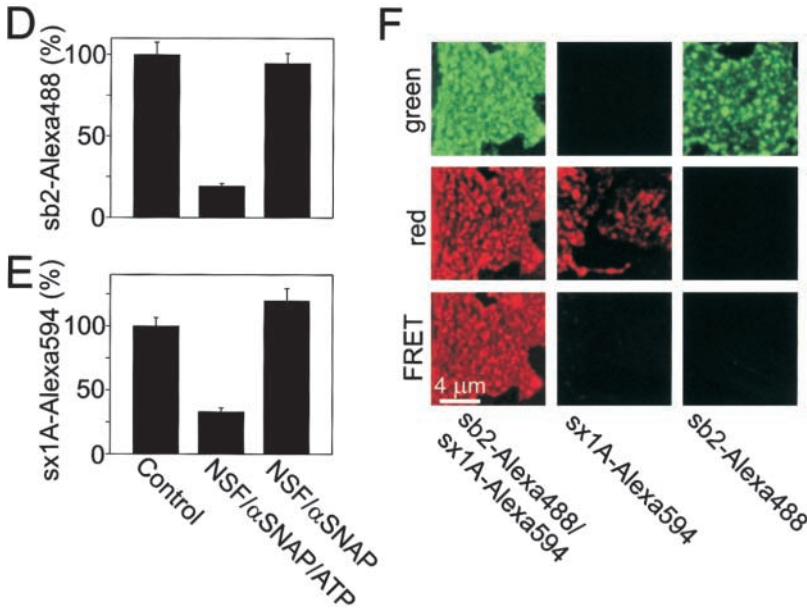
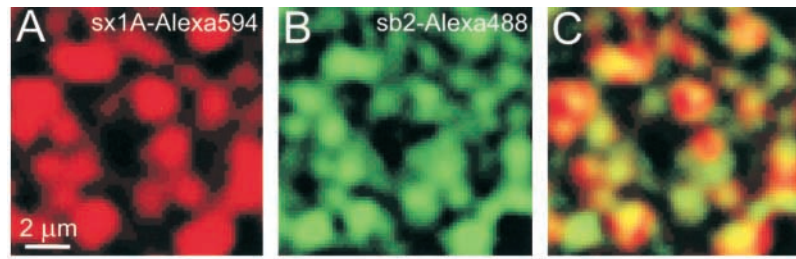
clostridial neurotoxins to the binding reaction in order to investigate whether binding is dependent only on endogenous syntaxin or whether endogenous SNAP-25 is also involved. As shown in Fig. 2 G, addition of either the syntaxin-specific BoNT/C1 or the SNAP-25-specific BoNT/E light chains to the binding reaction largely interfered with synaptobrevin binding, the residual binding activity being due to

the competition between toxin cleavage and synaptobrevin binding during the reaction, the latter resulting in the formation of toxin-resistant complexes. Thus, it appears that synaptobrevin needs both syntaxin and SNAP-25 for binding, corroborating the *in vitro* data, and showing that binding is limited by syntaxin but not by SNAP-25. If there is indeed excess binding capacity of SNAP-25, one would predict that not only overexpression of syntaxin but also the addition of exogenous syntaxin should increase synaptobrevin binding. To test this prediction, we measured binding of synaptobrevin (labeled with Alexa488) after incubating the membrane sheets with syntaxin 1A-Alexa594 that is supposed to form in conjunction with excess SNAP-25 the acceptor sites for exogenous synaptobrevin. As expected, a close correlation between the amount of bound syntaxin and synaptobrevin was observed (Fig. 2 H).

The characteristics of synaptobrevin binding suggests that the protein binds to the membranes by SNARE-specific interactions and that binding requires both SNAP-25 and syntaxin. These findings are best explained if synaptobrevin recruits endogenous syntaxin and SNAP-25 to form cis-core complexes in the membrane. We performed several control experiments to confirm this interpretation. Core complex formation would imply that exogenously added syntaxin and synaptobrevin bind to the same site. Fig. 3 (A–C) show that this is indeed the case:  $92 \pm 2\%$  of all synaptobrevin-Alexa488 spots colocalized with syntaxin-Alexa594 spots, and  $84 \pm 4\%$  of the syntaxin spots colocalized with synaptobrevin spots. Furthermore, a fluorescence resonance energy transfer (FRET) signal was observed between synaptobrevin 2-Alexa488 and syntaxin 1A-Alexa594 that occurs only when the two fluorophores are separated by distances smaller than 3–10 nm (Fig. 3 F). Thus, FRET demonstrates that both proteins are incorporated simultaneously into the same complex. To confirm that the complexes formed by the recombinant SNAREs are SNARE complexes, the membranes were incubated with  $\alpha$ SNAP/NSF-ATP that disassembles SNARE complexes. As expected, both exogenously bound syntaxin 1A-Alexa594 or synaptobrevin 2-Alexa488 were removed from the membranes in an ATP-dependent manner (Fig. 3, D and E).

### Syntaxin and SNAP-25 in the plasma membrane are free but form endogenous SNARE complexes upon prolonged incubation

Thus far, the results suggest that SNAREs in the plasma membrane are reactive and readily form SNARE complexes with exogenous recombinant SNAREs. However, we cannot exclude that a significant pool of the endogenous SNAREs is present in preformed complexes that do not bind exogenous SNAREs. To test for this possibility, we incubated freshly prepared membranes with an excess of BoNT/C1. This toxin only cleaves syntaxin that is uncomplexed (Blasi et al., 1993) or part of binary complexes with SNAP-25 (C. Schütte, personal communication) whereas syntaxin in core complexes is cleavage-resistant (Hayashi et al., 1994; Otto et al., 1997). The efficiency of syntaxin cleavage was assayed using indirect immunofluorescence. As shown in Fig. 4, BoNT/C1 treatment causes an almost quantitative removal of syntaxin, whereas SNAP-25 remains unchanged, demonstrating that no ternary complexes are present.



### Figure 3. Syntaxin 1A–Alexa594 and synaptobrevin 2–Alexa488 form SNARE complexes on inside-out membrane sheets.

(A–C) Membrane sheets reacted simultaneously with syntaxin 1A–Alexa594 (red channel) and synaptobrevin 2–Alexa488 (green channel). (D and E) NSF removes bound syntaxin 1 and synaptobrevin 2 from inside-out membranes. Membrane sheets reacted for 50 min with either synaptobrevin 2–Alexa488 (D) or syntaxin 1A–Alexa594 (E) were incubated with 0.5  $\mu\text{g}/\mu\text{l}$   $\alpha\text{SNAP}$  and 0.1  $\mu\text{g}/\mu\text{l}$  NSF during the last 10 min of the binding reaction, either in the presence of ATP or in the absence of ATP (including 1 mM EDTA). (F) Membrane sheets were reacted with syntaxin 1A–Alexa594 and synaptobrevin 2–Alexa488 either together (left) or alone (middle, right) and viewed in the green, red or the FRET channel. When both labeled proteins are bound a strong signal is detected in the FRET channel.

Because the plasma membrane contains not only high levels of syntaxin and SNAP-25 but also considerable amounts of synaptobrevin, the lack of cis-core complexes can be explained in two ways. First, the SNAREs are physically separated from each other, thus excluding close-range interactions as required for core complex formation. Second, the membrane-resident SNAREs are capable of forming core complexes but complex formation is slow; thus, NSF-driven disassembly dominates the equilibrium. According to the latter scenario one would expect that prolonged incubation of the membrane sheets in the absence of ATP-NSF and  $\alpha\text{-SNAP}$  would allow for the formation of toxin-resistant core complexes. As shown in Fig. 4 E, this was indeed the case. When the membrane sheets were incubated for 60 min before toxin treatment, syntaxin became largely cleavage resistant, suggesting that the majority of syntaxin forms core complexes.

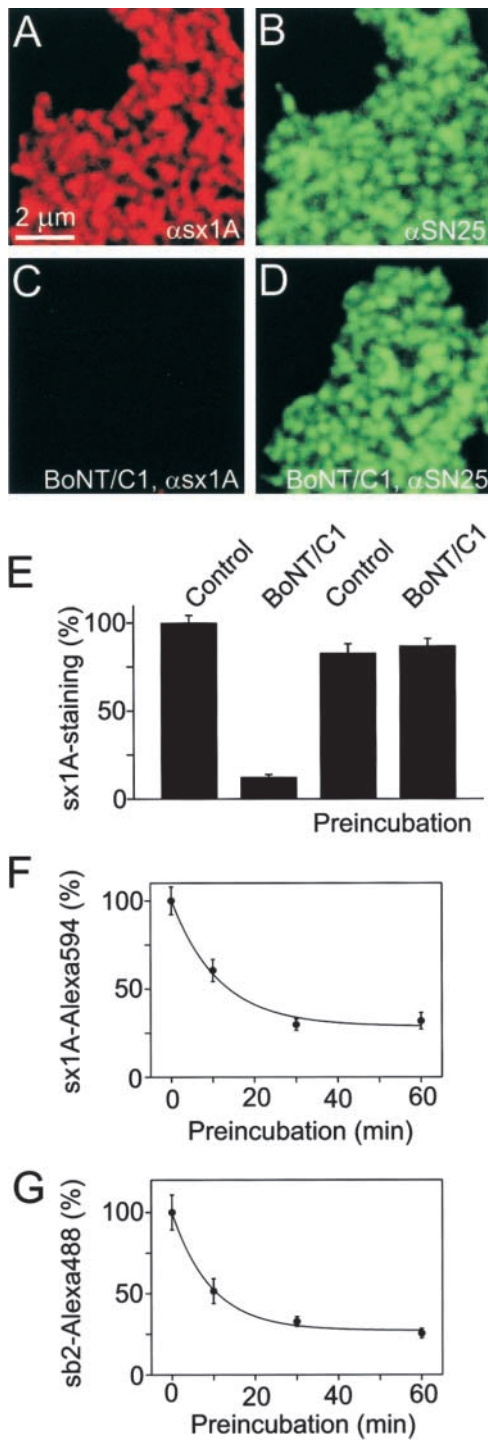
To confirm that binding of exogenous syntaxin and synaptobrevin only occurs when the endogenous SNAREs are not complexed, we performed binding experiments with fluorescently labeled syntaxin and synaptobrevin after various preincubation times. As expected, a time-dependent decrease of binding was observed for both proteins (Fig. 4, F and G). For synaptobrevin, the decrease could at least partially be prevented when the sheets were treated with TeNT during the preincubation time (unpublished data), demonstrating that the binding site for exogenous synaptobrevin is competed for by the endogenous protein during preincubation.

Although these results exclude that there are significant amounts of core complexes, it is still possible that at least part

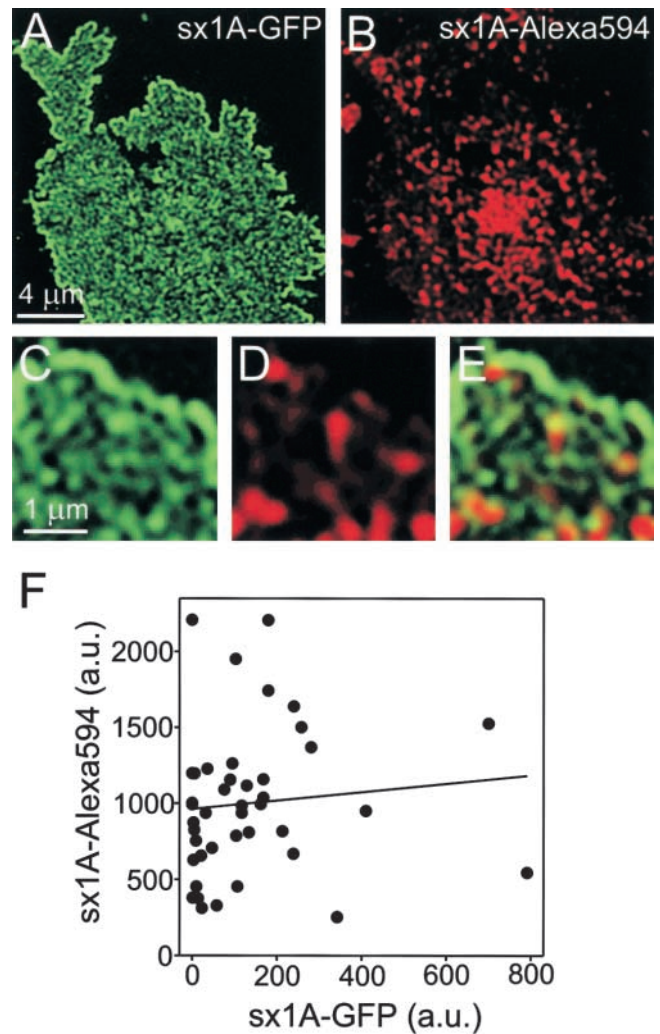
of the endogenous syntaxin is associated with SNAP-25. As discussed above, such binary complexes are required for synaptobrevin binding in *in vitro* assembly reactions, and they may provide a convenient explanation for the dependence of synaptobrevin binding on both endogenous syntaxin and SNAP-25. If such binary complexes are indeed present, one would expect that an increase of endogenous syntaxin should progressively compete with the binding of exogenously added syntaxin, resulting in a negative correlation between endogenous syntaxin and the binding of exogenous syntaxin. To test for this possibility, membrane sheets from cells overexpressing syntaxin 1A–GFP were reacted with syntaxin 1A–Alexa594 (Fig. 5). When the syntaxin 1A–GFP fluorescence was plotted against syntaxin 1A–Alexa594, no correlation was found (Fig. 5 F), suggesting that no significant pool of stable binary complexes of syntaxin and SNAP-25 is present. This is consistent with our observation that BoNT/C treatment does not increase the binding sites for syntaxin (see above). Furthermore, overlap between GFP-syntaxin and bound exogenous syntaxin was low (Fig. 5, A–E). We conclude that SNAREs in native plasma membranes do not form stable SNARE complexes. Rather, they are free and active in their capability to bind to their partner SNAREs.

## Discussion

In this study, we have used several approaches to show that in intact plasma membranes the SNAREs SNAP-25 and syntaxin are largely active in a constitutive manner. Thus,



**Figure 4. Endogenous syntaxin 1 is quantitatively cleaved by BoNT/C1 on freshly isolated inside-out membrane sheets but not after prolonged incubation suggesting formation of SNARE core complexes over time.** (A–D) Membrane sheets treated for 45 min in the presence (C and D) or absence (A and B) of 5  $\mu$ M BoNT/C1 were immunostained for syntaxin 1A/B (A and C) and SNAP-25 (B and D). (E) Quantification of syntaxin-immunostaining intensity on membrane sheets as shown in A and C and membrane sheets that were incubated for 1 h before BoNT/C1 treatment. Binding of syntaxin 1A–Alexa594 (F) and synaptobrevin 2–Alexa488 (G) to membrane sheets that were preincubated at 37°C in buffer for 0, 10, 30, or 60 min before addition of the labeled proteins.



**Figure 5. Binding of exogenous syntaxin 1A is not affected by syntaxin overexpression.** Syntaxin 1A–Alexa594 binding was measured on membrane sheets from cells expressing syntaxin 1A–GFP. (A) Syntaxin 1A–GFP (green channel). (B) Syntaxin 1A–Alexa594 (red channel). (C and D) Magnified views from A and B. (E) Overlap from C and D. (F) Plotting green against red fluorescence intensity in random images obtained from membrane sheets as shown in A and B.

the SNAREs are not downregulated by interacting with each other or with other proteins. These results are unexpected in the light of many reports describing upstream control of SNAREs by other proteins. Our data do not exclude that SNAREs are regulated before fusion. However, they clearly document that such control mechanism do not operate by inactivating the intrinsic ability of the respective SNARE motifs to enter core complexes, at least not in a cis-configuration. Furthermore, the binding properties of membrane-resident SNAREs resemble those determined previously for SNAREs in solution (for review see Brunger, 2001), confirming that the SNARE motifs are solely responsible for these interactions.

Our data revealed a detailed picture about the status of SNAREs in the plasma membrane that is summarized in Fig. 6. When membranes are freshly prepared, SNAP-25 and syntaxin are present in separate clusters. Furthermore, the

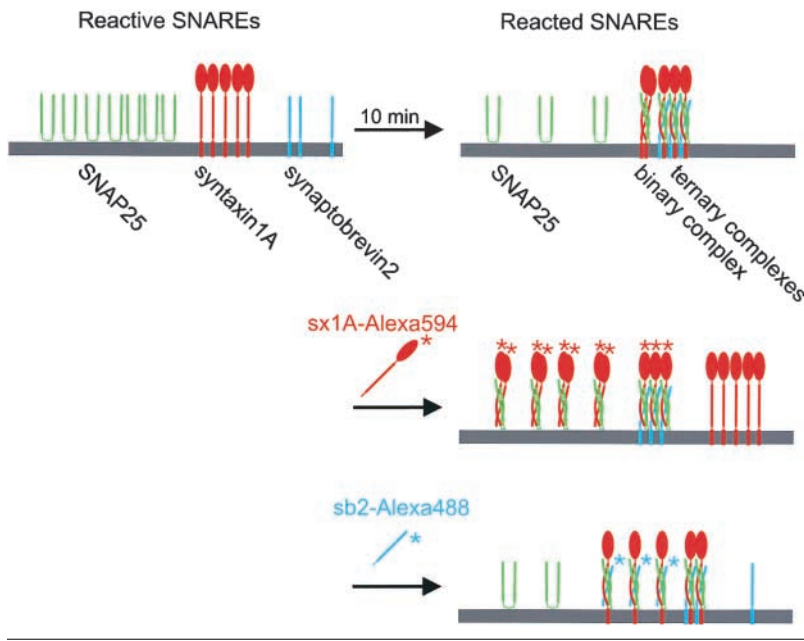


Figure 6. **Working model: in the native plasma membrane the SNAREs are reactive.** When isolated plasma membrane sheets age, the reactive endogenous SNAREs form binary and ternary complexes. Reactive endogenous SNAREs also form SNARE complexes with added recombinant SNAREs.

plasma membrane contains a pool of synaptobrevin that is also organized in clusters (unpublished data) and is not in complexes with its partner SNAREs. This conclusion is supported by (a) the observation that all SNAREs are sensitive to cleavage by clostridial neurotoxins that cannot attack their substrates in core complexes, and (b) our finding that the endogenous SNAREs readily bind exogenously added partner SNAREs in a specific and predictable manner.

Our data allow us to rule out that SNARE core complexes are present to a measurable degree in freshly prepared membranes. Similarly, it is unlikely that binary complexes between syntaxin and SNAP-25 as defined by *in vitro* binding assays are present. Such binary complexes are structurally similar to ternary complexes with the exception that the position of synaptobrevin is replaced by a second syntaxin molecule (Fasshauer et al., 1997a) and that the NH<sub>2</sub>- and COOH-terminal ends of the helical bundle are less structured (Margittai et al., 2001). Our conclusion is based on the observation that even strong overexpression of syntaxin does not reduce binding of exogenous syntaxin to SNAP-25. Furthermore, quantitative removal of endogenous syntaxin by BoNT/C1 does not increase binding of exogenous syntaxin which would be expected if significant proportions of SNAP-25 were complexed with endogenous syntaxin.

Intriguingly, there is some evidence for a loose connection between syntaxin and SNAP-25, or at least for a ready availability of SNAP-25 for the formation of syntaxin-dependent complexes. This is shown by our finding that exogenously added synaptobrevin recruits endogenous SNAP-25 into core-complexes at sites where syntaxin is clustered. SNAP-25 is linked to the membrane by palmitoyl anchors, and may thus be more mobile than syntaxin with its transmembrane domain that in addition tends to form homooligomers (Laage et al., 2000). Although both proteins reside in clusters, those of SNAP-25 are more numerous and appear more diffusive than syntaxin clusters with which they partially overlap (Lang et al., 2001). Hence, SNAP-25 is more abun-

dant in the membrane than syntaxin, which is also supported by our finding that syntaxin but not SNAP-25 is limiting in creating acceptor sites for synaptobrevin. Docked granules do not contribute significantly to these clusters, as <10% of the membrane sheets retain granules. Furthermore, as in these cases, the density of granules is at least fourfold lower than that of the SNARE clusters (Lang et al., 2001; unpublished data).

Ready availability of mobile endogenous SNAP-25 may also explain why recombinant SNAP-25 did not bind to the membranes (unpublished data). If syntaxin is indeed uncomplexed, one would expect that exogenously added SNAP-25 binds to the endogenous syntaxin. However, complex formation of exogenous SNAP-25 requires the simultaneous recruitment of two syntaxin molecules, and may thus be too slow, particularly when considering that the local concentration of endogenous SNAP-25 is probably far higher than that of exogenously added SNAP-25 (which like the other SNAREs was added at a concentration of 4  $\mu$ M).

Although the SNAREs are not present in binary or ternary SNARE complexes in freshly prepared membranes, such complexes form when the membranes age. In a live cell, a formed *cis*-SNARE complex would be disassembled by  $\alpha$ SNAP/NSF action to regenerate reactive SNAREs. Segregation of SNAREs into microdomains (Lang et al., 2001) may be an efficient process to keep the rate of unproductive SNARE complex formation low, allowing for NSF to maintain all SNAREs in a disassembled state under steady-state conditions. Because SNARE complex disassembly requires ATP hydrolysis, high turnover rates of SNARE complex assembly and disassembly would be unfavorable for the cell. As previously reported, syntaxin and SNAP-25 clusters overlap only partially (Lang et al., 2001). Low overlap between overexpressed syntaxin 1A-GFP, reflecting the endogenous syntaxin, and syntaxin 1A-Alexa594, reflecting the endogenous SNAP-25, are consistent with the idea that syntaxin and SNAP-25 are segregated at least in part in the mem-

brane, and the same holds true for synaptobrevin (unpublished data). Similarly, SDS-resistant SNARE complexes were observed in temperature-sensitive NSF mutants of *Drosophila* only at the restrictive temperature (Sanyal et al., 2001), supporting our notion that core complexes can only accumulate when NSF is inactive or when the ATP/ADP ratio is reduced.

An obvious question that arises is how is exocytic activity affected by the artificial generation of SNARE complexes in *in vitro* fusion assays? Our study shows that ternary SNARE complexes form when the membranes are either preincubated or reacted with recombinant SNAREs. It is well established that reactive SNAREs are required for exocytosis but, as outlined further below, the SNAREs are thought to be activated during vesicle docking by a priming reaction. When inside-out membrane sheets of PC12 cells were preincubated for 60 min at 37°C, exocytic activity of secretory granules that are associated with the plasma membrane decreased to low levels (Avery et al., 2000), with the most dramatic decrease occurring in the first few minutes after cell disruption (unpublished data), although priming conditions were maintained during the incubation. Although this decrease may also be caused by non-SNARE-dependent factors, a direct inhibitory action of exogenously added SNAREs on exocytosis has been described previously (Chen et al., 2001; Scales et al., 2000). When permeabilized PC12 cells were incubated with recombinant syntaxin 1A or synaptobrevin 2, exocytic activity, as measured by secretion of radioactively labeled norepinephrine, was blocked (Scales et al., 2000). However, in these experiments, only the small pool of SNAREs engaged in exocytosis was probed whose properties may be different.

The finding that membrane-resident SNAREs spontaneously form SNARE complexes during membrane isolation and that complex formation is associated with loss of exocytic activity raises important questions about the interpretation of data acquired from *in vitro* fusion assays such as exocytosis in permeabilized cells (Holz et al., 1989; Robinson and Martin, 1998; Chen et al., 2001), or homotypic fusion of isolated yeast vacuoles (Haas et al., 1994; Wickner and Haas, 2000) or mammalian endosomes (McBride et al., 1999). From these assays, detailed models were put forward describing the sequence of steps leading to membrane fusion. It is currently believed that membranes first are docked/tethered and primed before they can undergo fusion. Priming requires  $\alpha$ SNAP/NSF and ATP in permeabilized cells (Banerjee et al., 1996) and in the yeast vacuole fusion system (Mayer and Wickner, 1997), suggesting that SNAREs need to be activated for membrane fusion. However, the need for SNARE activation during priming may be an experimental artifact, as during the preparation of the material the SNAREs are expected to react with each other to form inactive cis-complexes. Hence, priming of SNAREs may not be part of a sequence that leads to fusion, but they represent the normal state of the proteins in live cells.

Our finding that plasma membrane-resident SNAREs are predominantly active suggests that the availability of reactive SNAREs is not regulated by controlling SNARE activity itself. Obviously, probing endogenous SNAREs with recombinant proteins will only measure the capacity of the

SNAREs to form cis-complexes, and it cannot be excluded that the ability to enter trans-complexes is regulated in an unknown fashion that is not uncovered by our assays. At least we can safely conclude that the availability of the reactive SNARE motifs per se is not subject to regulation. Rather, regulation may occur at the level of docking and at the level of trans-complexes whose activity may be modulated by proteins such as synaptotagmin or complexin.

## Materials and methods

### Cell culture and transfection

PC12 cells (clone 251; Heumann et al., 1983) were maintained and propagated as described (Lang et al., 1997). Transfection of PC12 cells with syntaxin 1A-EGFP (Lang et al., 2001) was performed essentially as described previously (Lang et al., 1997).

### Antibodies

For the detection of syntaxin 1A, a mouse monoclonal antibody was used (HPC-1; Barnstable et al., 1985). For detection of SNAP-25, a rabbit polyclonal sera was used (Aguado et al., 1996). Secondary antibodies used were Cy2-coupled goat-anti-rabbit and Cy3-coupled goat-anti-mouse (Dianova). The following mouse monoclonal antibodies were used in the binding studies: anti-Rab3a/b (Cl 42.1; Synaptic Systems), anti-synaptobrevin 2 (Cl 69.1; Edelmann et al., 1995), and anti-SNAP-25 (Cl 71.1; Xu et al., 1999).

### Recombinant proteins

The following expression constructs have been described before: synaptobrevin 2 (1-96) S28C (Margittai et al., 2001), SNAP-25 (Fasshauer et al., 1999), syntaxin 1A (cytosolic fragment 1-262; Pabst et al., 2000), and  $\alpha$ SNAP and NSF (Hanson et al., 1995). A single cysteine mutant of syntaxin 1A (1-262) C145S, S171C was generated by primer-mediated mutagenesis as described previously (Margittai et al., 2001). The construct was subcloned into pET28a (Novagen) into the Nde/Xho restriction site. Correctness of the DNA sequence was confirmed by DNA sequencing.

All recombinant SNAREs were expressed as His<sub>6</sub>-tagged fusion proteins and purified by Ni<sup>2+</sup>-Agarose. The proteins were dialyzed against KGLu buffer (20 mM Hepes, pH 7.2, 120 mM potassium glutamate, 20 mM potassium acetate, 1 mM dithiothreitol). The tags were removed using thrombin and proteins were further purified using Mono-Q or Mono-S columns on an FPLC system (Pharmacia). For fluorescence labeling DTT was removed by size-exclusion chromatography (Sephadex G50 superfine material, Amersham Biosciences) and syntaxin 1A (1-262) S171C and synaptobrevin (1-96) S28C were incubated for 3 h on ice with a 10-fold excess of maleimide-coupled Alexa488 or Alexa594 (Molecular Probes). The reaction was stopped by the addition of 10 mM DTT, and labeled proteins were separated from free dye by size-exclusion chromatography (Sephadex G50 superfine material; Amersham Biosciences) in KGLu buffer. Labeling efficiencies ranged from 50 to 100%. Recombinant  $\alpha$ SNAP was expressed as a GST fusion protein and purified using glutathione Sepharose. The tag was removed using thrombin and the protein was further purified using a Mono-Q column on an FPLC system (Amersham Biosciences). BoNT/C and E and TeNT light chains, a gift from Heiner Niemann (Medizinische Hochschule Hanover, Hanover, Germany), and NSF were expressed as His<sub>6</sub>-tagged fusion proteins and purified by Ni<sup>2+</sup>-Agarose. Proteins were dialyzed against KGLu buffer. In the case of NSF, during all steps, 1 mM ATP and 2 mM MgCl<sub>2</sub> were present.

### Binding studies

For preparation of membrane sheets, cells were grown on poly-L-lysine-coated coverslips and disrupted as described previously (Avery et al., 2000) using a 100-ms ultrasound pulse in ice-cold sonication-buffer (20 mM Hepes, pH 7.2, 120 mM potassium glutamate, 20 mM potassium acetate, 10 mM EGTA, 2 mM MgATP, and 0.5 mM dithiothreitol). Under these conditions, the majority of the generated membranes are devoid of secretory granules. The membrane sheets were then reacted with the proteins indicated at 37°C in KGLu-BSA (KGLu containing 4 mM MgCl<sub>2</sub>, 2 mM ATP and 3% BSA) at 37°C in a humid chamber for 30–60 min (half maximal labeling was observed after ~5 min; unpublished data). The concentration of the labeled fluorescent SNAREs was always 4  $\mu$ M. When clostridial neurotoxin light chains or an inhibitory antibody was used, these reagents were added simultaneously with the labeled probe. Membrane sheets were then washed two to three times at room temperature in KGLu-BSA for



40–80 min. Membrane sheets were washed once in PBS (150 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4), fixed for at least 2 h in 4% PFA, washed in PBS, and imaged in PBS containing 4% of a 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene (TMA-DPH) saturated PBS solution. TMA-DPH visualizes phospholipid membranes and allows for identifying membrane sheets without any information of the fluorescence in the other channels, hence avoiding preferential selection of membrane sheets according to their staining intensity. Using TMA-DPH further avoided strong bleaching of the green or the red label due to the positioning of the membranes into the field of the camera and the focusing procedure. All preincubation steps with or without recombinant proteins were performed at 37°C in KGLu-BSA. In some experiments, reacted membrane sheets were immunostained by performing the protocol described below, with the following changes: anti-SNAP-25 was diluted 1:100; goat-anti-rabbit-Cy2 was diluted 1:200; incubation time with the primary antibody was 2 h; and incubation time with the secondary antibody was 90 min.

### Quantitation of fluorescence signals

For comparative quantitation of fluorescence intensity, membrane sheets were identified in the TMA-DPH pictures, each containing up to 15–20 individual membrane sheets. Up to five areas of 5–10 μm<sup>2</sup> each covering several dozens of clusters were placed randomly on membrane sheets and then transferred to the other channels with corrections being made to avoid obvious artefacts such as highly fluorescent contaminating particles that were occasionally seen. In each area, the overall fluorescence intensity was quantitated. Local background was measured in an area outside the membrane sheets and subtracted. For each condition, 23–60 (mean = 37) membrane sheets were analysed. Values are given as mean ± SEM.

To determine colocalization of spots in two channels, we used a procedure similar to that described previously (Lang et al., 2001). Circles were centered around randomly selected individual spots (25 each per individual membrane sheet) in one channel (Fig. 1) and then transferred to the second channel. If the fluorescence intensity maximum of a corresponding spot in the second channel was within 200 nm of the spot in the first channel, it was rated as colocalized. To correct for accidental colocalization, the image of the first channel was superimposed with a mirror image of the second channel, and colocalization was determined as above. The background values ranged between 12 and 22%. Background correction was performed according to the following formula: real colocalization = (measured colocalization – background colocalization)/(1 – background colocalization/100). This algorithm takes into account that the contribution of background colocalization is inversely related to the real colocalization. For each experimental condition, we analyzed ten individual membrane sheets. Values are given as mean ± SEM.

### Immunofluorescence

Before immunostaining of the membrane sheets, all antibodies were diluted (anti-syntaxin 1A/B 1:50, anti-SNAP-25 1:100, goat-anti-rabbit-Cy2 1:200, goat-anti-mouse-Cy3 1:200) into PBS (150 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4) containing 1% (wt/vol) BSA, incubated for 45 min at room temperature, and centrifuged for 10 min at 13000 g. Membrane sheets freshly prepared or incubated for 1 h in KGLu-BSA were incubated for 45 min in KGLu-BSA containing 5 μM BoNT/C. Samples were fixed for 12 h at 4°C in 4% paraformaldehyde in PBS, quenched for 20 min in PBS containing 50 mM NH<sub>4</sub>Cl, and washed three times in PBS for 10 min each. They were then incubated for 40 min with the primary antibody. Subsequently, sheets were washed two times in PBS for 10 min each, followed by a 40-min incubation with the secondary antibodies. Membrane sheets were washed two times in PBS for 15 min and once for 40 min, and then imaged in PBS containing 4% of a TMA-DPH-saturated PBS solution. For each condition, 21–25 membrane sheets were analyzed. Values are given as mean ± SEM.

### Fluorescence microscopy

Membrane sheets were analysed using a Zeiss Axiovert 100 TV fluorescence microscope with a 100× 1.4 NA plan achromate objective. For imaging, we used a back-illuminated frame transfer CCD camera (2× 512 × 512-EEV chip, 13 × 13 μm pixel size; Princeton Instruments, Inc.) with a magnifying lens (1.6× Optovar) to avoid spatial undersampling by the large pixels. GFP-, Cy2-, and Alexa488 fluorescence was detected using excitation filter BP 480/40, BS 505, and emission filter BP 527/30 (AHF Analysentechnik AG), for Cy3 and Alexa594 fluorescence excitation filter BP 565/30, BS 595, and emission BP 645/75 was used (AHF Analysentechnik AG). FRET was detected using BP 480/40, BS 505, and emission filter BP 645/75 blocked against green light (AHF analysentechnik AG). TMA-DPH fluorescence was detected using Zeiss filter set 02 (excitation filter G

365, BS 395, and emission LP 420). Images were analysed with MetaMorph (Universal Imaging Corporation). The images shown in Fig. 1–3 (A–C) and 5 were resampled with double resolution and deconvoluted by the theoretical point spread function of the microscope. This theoretical point spread function was calculated numerically, using the wavelength, aperture and magnification. We employed the nonlinear Richardson-Lucy deconvolution algorithm (Van Kempen et al., 1997).

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## References

- Aguado, F., G. Majo, B. Ruiz-Montasell, J.M. Canals, A. Casanova, J. Marsal, and J. Blasi. 1996. Expression of synaptosomal-associated protein SNAP-25 in endocrine anterior pituitary cells. *Eur. J. Cell Biol.* 69:351–359.
- Antonin, W., D. Fasshauer, S. Becker, R. Jahn, and T.R. Schneider. 2002. Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. *Nat. Struct. Biol.* 9:107–111.
- Avery, J., R. Jahn, and J.M. Edwardson. 1999. Reconstitution of regulated exocytosis in cell-free systems: a critical appraisal. *Annu. Rev. Physiol.* 61:777–807.
- Avery, J., D.J. Ellis, T. Lang, P. Holroyd, D. Riedel, R.M. Henderson, J.M. Edwardson, and R. Jahn. 2000. A cell-free system for regulated exocytosis in PC12 cells. *J. Cell Biol.* 148:317–324.
- Banerjee, A., V.A. Barry, B.R. DasGupta, and T.F. Martin. 1996. N-Ethylmaleimide-sensitive factor acts at a prefusion ATP-dependent step in Ca<sup>2+</sup>-activated exocytosis. *J. Biol. Chem.* 271:20223–20226.
- Barnstable, C.J., R. Hofstein, and K. Akagawa. 1985. A marker of early amacrine cell development in rat retina. *Brain Res.* 352:286–290.
- Binz, T., J. Blasi, S. Yamasaki, A. Baumeister, E. Link, T.C. Sudhof, R. Jahn, and H. Niemann. 1994. Proteolysis of SNAP-25 by types E and A botulinum neurotoxins. *J. Biol. Chem.* 269:1617–1620.
- Blasi, J., E.R. Chapman, S. Yamasaki, T. Binz, H. Niemann, and R. Jahn. 1993. Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. *EMBO J.* 12:4821–4828.
- Brunger, A.T. 2001. Structural insights into the molecular mechanism of calcium-dependent vesicle-membrane fusion. *Curr. Opin. Struct. Biol.* 11:163–173.
- Bruns, D., and R. Jahn. 2002. Molecular determinants of exocytosis. *Pflugers Arch.* 443:333–338.
- Chen, Y.A., and R.H. Scheller. 2001. SNARE-mediated membrane fusion. *Nat. Rev. Mol. Cell Biol.* 2:98–106.
- Chen, Y.A., S.J. Scales, and R.H. Scheller. 2001. Sequential SNARE assembly underlies priming and triggering of exocytosis. *Neuron.* 30:161–170.
- Edelmann, L., P.I. Hanson, E.R. Chapman, and R. Jahn. 1995. Synaptobrevin binding to synaptophysin: a potential mechanism for controlling the exocytotic fusion machine. *EMBO J.* 14:224–231.
- Fasshauer, D., D. Bruns, B. Shen, R. Jahn, and A.T. Brunger. 1997a. A structural change occurs upon binding of syntaxin to SNAP-25. *J. Biol. Chem.* 272:4582–4590.
- Fasshauer, D., H. Otto, W.K. Eliason, R. Jahn, and A.T. Brunger. 1997b. Structural changes are associated with soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor complex formation. *J. Biol. Chem.* 272:28036–28041.
- Fasshauer, D., W. Antonin, M. Margittai, S. Pabst, and R. Jahn. 1999. Mixed and non-cognate SNARE complexes. Characterization of assembly and biophysical properties. *J. Biol. Chem.* 274:15440–15446.
- Haas, A., B. Conradt, and W. Wickner. 1994. G-protein ligands inhibit in vitro reactions of vacuole inheritance. *J. Cell Biol.* 126:87–97.
- Hanson, P.I., H. Otto, N. Barton, and R. Jahn. 1995. The N-ethylmaleimide-sensitive fusion protein and alpha-SNAP induce a conformational change in syntaxin. *J. Biol. Chem.* 270:16955–16961.
- Hanson, P.I., R. Roth, H. Morisaki, R. Jahn, and J.E. Heuser. 1997. Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell.* 90:523–535.
- Hayashi, T., H. McMahon, S. Yamasaki, T. Binz, Y. Hata, T.C. Sudhof, and H. Niemann. 1994. Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. *EMBO J.* 13:5051–5061.
- Hayashi, T., S. Yamasaki, S. Nauenburg, T. Binz, and H. Niemann. 1995. Disassembly of the reconstituted synaptic vesicle membrane fusion complex in

- vitro. *EMBO J.* 14:2317–2325.
- Heumann, R., V. Kachel, and H. Thoenen. 1983. Relationship between NGF-mediated volume increase and “priming effect” in fast and slow reacting clones of PC12 pheochromocytoma cells. Role of cAMP. *Exp. Cell Res.* 145:179–190.
- Holz, R.W., M.A. Bittner, S.C. Peppers, R.A. Senter, and D.A. Eberhard. 1989. MgATP-independent and MgATP-dependent exocytosis. Evidence that MgATP primes adrenal chromaffin cells to undergo exocytosis. *J. Biol. Chem.* 264:5412–5419.
- Jahn, R., and T.C. Sudhof. 1999. Membrane fusion and exocytosis. *Annu. Rev. Biochem.* 68:863–911.
- Laage, R., J. Rohde, B. Brosig, and D. Langosch. 2000. A conserved membrane-spanning amino acid motif drives homomeric and supports heteromeric assembly of presynaptic SNARE proteins. *J. Biol. Chem.* 275:17481–17487.
- Lang, T., D. Bruns, D. Wenzel, D. Riedel, P. Holroyd, C. Thiele, and R. Jahn. 2001. SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis. *EMBO J.* 20:2202–2213.
- Lang, T., I. Wacker, J. Steyer, C. Kaether, I. Wunderlich, T. Soldati, H.H. Gerdes, and W. Almers. 1997. Ca<sup>2+</sup>-triggered peptide secretion in single cells imaged with green fluorescent protein and evanescent-wave microscopy. *Neuron.* 18:857–863.
- Lonart, G., and T.C. Sudhof. 2000. Assembly of SNARE core complexes prior to neurotransmitter release sets the readily releasable pool of synaptic vesicles. *J. Biol. Chem.* 275:27703–27707.
- Lupashin, V.V., and M.G. Waters. 1997. t-SNARE activation through transient interaction with a rab-like guanosine triphosphatase. *Science.* 276:1255–1258.
- Margittai, M., D. Fasshauer, S. Pabst, R. Jahn, and R. Langen. 2001. Homo- and heterooligomeric SNARE complexes studied by site-directed spin labeling. *J. Biol. Chem.* 276:13169–13177.
- Mayer, A., and W. Wickner. 1997. Docking of yeast vacuoles is catalyzed by the Ras-like GTPase Ypt7p after symmetric priming by Sec18p (NSF). *J. Cell Biol.* 136:307–317.
- McBride, H.M., V. Rybin, C. Murphy, A. Giner, R. Teasdale, and M. Zerial. 1999. Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. *Cell.* 98:377–386.
- Munson, M., X. Chen, A.E. Cocina, S.M. Schultz, and F.M. Hughson. 2000. Interactions within the yeast t-SNARE Sso1p that control SNARE complex assembly. *Nat. Struct. Biol.* 7:894–902.
- Otto, H., P.I. Hanson, and R. Jahn. 1997. Assembly and disassembly of a ternary complex of synaptobrevin, syntaxin, and SNAP-25 in the membrane of synaptic vesicles. *Proc. Natl. Acad. Sci. USA.* 94:6197–6201.
- Pabst, S., J.W. Hazzard, W. Antonin, T.C. Sudhof, R. Jahn, J. Rizo, and D. Fasshauer. 2000. Selective interaction of complexin with the neuronal SNARE complex. Determination of the binding regions. *J. Biol. Chem.* 275:19808–19818.
- Pfeffer, S.R. 1999. Transport-vesicle targeting: tethers before SNAREs. *Nat. Cell Biol.* 1:E17–E22.
- Robinson, L.J., and T.F. Martin. 1998. Docking and fusion in neurosecretion. *Curr. Opin. Cell Biol.* 10:483–492.
- Sanyal, S., L.A. Tolar, L. Pallanck, and K.S. Krishnan. 2001. Genetic interaction between shibire and comatose mutations in *Drosophila* suggest a role for snap-receptor complex assembly and disassembly for maintenance of synaptic vesicle cycling. *Neurosci. Lett.* 311:21–24.
- Scales, S.J., Y.A. Chen, B.Y. Yoo, S.M. Patel, Y.C. Doung, and R.H. Scheller. 2000. SNAREs contribute to the specificity of membrane fusion. *Neuron.* 26:457–464.
- Schiavo, G., F. Benfenati, B. Poulain, O. Rossetto, P. Polverino de Laureto, B.R. DasGupta, and C. Montecucco. 1992. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature.* 359:832–835.
- Schiavo, G., C.C. Shone, M.K. Bennett, R.H. Scheller, and C. Montecucco. 1995. Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. *J. Biol. Chem.* 270:10566–10570.
- Sollner, T., M.K. Bennett, S.W. Whiteheart, R.H. Scheller, and J.E. Rothman. 1993. A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell.* 75:409–418.
- Sutton, R.B., D. Fasshauer, R. Jahn, and A.T. Brunger. 1998. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature.* 395:347–353.
- Tochio, H., M.M. Tsui, D.K. Banfield, and M. Zhang. 2001. An autoinhibitory mechanism for nonsyntaxin SNARE proteins revealed by the structure of Ykt6p. *Science.* 293:698–702.
- Van Kempen, G.M.P., L.J. Van Vliet, P.J. Verveer, and H.M.T. van der Voort. 1997. A quantitative comparison of image restoration methods for confocal microscopy. *J. Microsc.* 185:354–365.
- Washbourne, P., G. Schiavo, and C. Montecucco. 1995. Vesicle-associated membrane protein-2 (synaptobrevin-2) forms a complex with synaptophysin. *Biochem. J.* 305:721–724.
- Weber, T., B.V. Zemelman, J.A. McNew, B. Westermann, M. Gmachl, F. Parlati, T.H. Sollner, and J.E. Rothman. 1998. SNAREpins: minimal machinery for membrane fusion. *Cell.* 92:759–772.
- Wickner, W., and A. Haas. 2000. Yeast homotypic vacuole fusion: a window on organelle trafficking mechanisms. *Annu. Rev. Biochem.* 69:247–275.
- Xu, T., B. Rammner, M. Margittai, A.R. Artalejo, E. Neher, and R. Jahn. 1999. Inhibition of SNARE complex assembly differentially affects kinetic components of exocytosis. *Cell.* 99:713–722.
- Yang, B., M. Steegmaier, L.C. Gonzalez, Jr., and R.H. Scheller. 2000. nSec1 binds a closed conformation of syntaxin1A. *J. Cell Biol.* 148:247–252.