Evaluation of the Heterogeneous Reactivity of the Syntaxin Molecules on the Inner Leaflet of the Plasma Membrane

Dana Bar-On,^{1,2} Menachem Gutman,² Aviv Mezer,² Uri Ashery,¹ Thorsten Lang,³ and Esther Nachliel²

¹Department of Neurobiochemistry and ²Laser Laboratory for Fast Reactions in Biology, Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel, and ³Department of Neurobiology, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany

The soluble *N*-ethylmaleimide-sensitive fusion (NSF) attachment protein (SNAP) receptor (SNARE) protein syntaxin 1A forms nano-sized clusters (membrane rafts) on the plasma membrane (PM) that are in equilibrium with freely diffusing syntaxin molecules. SNARE-complex formation between syntaxin 1A and SNAP-25 (synaptosome-associated protein of 25 kDa) on the PM and synaptobrevin 2 on the vesicles (*trans*-SNAREs) is crucial for vesicle priming and fusion. This process might be impeded by the spontaneous accumulation of non-fusogenic *cis*-SNARE complexes formed when all three SNARE proteins reside on the PM. We investigated the kinetics of *cis*-SNARE complex assembly and disassembly and both exhibited biphasic behavior. The experimental measurements were analyzed through integration of differential rate equations pertinent to the reaction mechanism and through the application of a heuristic search for time constants and concentrations using a genetic algorithm. Reconstruction of the measurements necessitated the partitioning of syntaxin into two phases that might represent the syntaxin clusters and free syntaxin outside the clusters. The analysis suggests that most of the syntaxin in the clusters is concentrated in a nonreactive form. Consequently, *cis*-SNARE complex assembly in the clusters is substantially slower than outside the rafts. Interestingly, the clusters also mediate efficient disassembly of *cis*-SNARE complexes possibly attributable to the high local concentration of complexes in the clusters area that allows efficient disassembly by the enzymatic reaction of NSF.

Introduction

The fusion of biological membranes is one of the most fundamental processes in biology, essential for protein trafficking, hormone secretion, and neurotransmission. The core fusion machinery is the heterotrimeric soluble *N*-ethylmaleimide-sensitive fusion (NSF) attachment protein (SNAP) receptor complex, termed SNARE complex, which is crucial for the fusion of vesicles with the cell membrane (Fasshauer, 2003). The SNARE proteins involved in the fusion of synaptic vesicles are syntaxin-1A and a synaptosome-associated protein of 25 kDa (SNAP-25), which reside on the pre-synaptic plasma membrane (PM), and synaptobrevin (2/VAMP2), which resides on the vesicle membrane. When the vesicle is close enough to the PM, a *trans*-SNARE complex is formed that is believed to drive the fusion of vesicular and pre-

synaptic membranes (Jahn et al., 2003). After the fusion, the *trans*-SNARE complex transforms into a *cis*-SNARE complex, with all of its components located on the inner leaflet of the PM (Jahn et al., 2003). Uncoupled from the fusion process, when the three SNARE components are present on the PM, they can form *cis*-SNARE complexes spontaneously (Otto et al., 1997; Lang et al., 2002). The availability of the SNARE proteins for *trans*-SNARE complex formation depends on keeping the SNARE proteins in a reactive state (Littleton et al., 2001; Sanyal et al., 2001) and therefore might be impeded by the accumulation of *cis*-SNARE complex on the PM. The reactivity of the SNARE proteins can be maintained by disassembly of the *cis*-SNARE complexes back into their constituents, which is executed by the combined action of the ATPase enzyme NSF and its cofactor α SNAP (Söllner et al., 1993).

We recently described the kinetics of *cis*-SNARE complex assembly and disassembly on the native PM sheets of pheochromocytoma 12 (PC12) cells (Bar-On et al., 2008). In the current study, we extend the experimental system to monitor the quasi-equilibrium between the two processes. In terms of chemical kinetics, accumulation of *cis*-SNARE complexes is an energy-dissipating process. Formation of the complexes is a spontaneous forward reaction, and their disassembly is achieved by an energy-consuming catalytic reaction driven by the ATPase NSF. Accordingly, under *in vivo* and *ex vivo* conditions, the *cis*-SNARE complexes population is far from thermodynamic control and is under strict kinetics control. The quasi-equilibrium dynamics of the *cis*-SNARE complexes can be reconstructed through the equations and the rate constants of the assembly and disassembly processes. In the present study, we subjected our past and present experimental

Received Feb. 11, 2009; revised May 27, 2009; accepted July 6, 2009.

This work was supported by Israel Science Foundation Grant 1211/07 (U.A.) and National Institutes of Health Grant R01 NS053978 (U.A.). A.M. is a recipient of a Machiah Foundation fellowship. The experimental work described in this manuscript was performed in the Department of Neurobiology, Max Planck Institute for Biophysical Chemistry (Göttingen, Germany). We thank Prof. Reinhard Jahn for his constant support and Dr. Dirk Fasshauer and Dr. Ulrike Winter for the generous gift of purified disassembly proteins that were essential for the completion of the study. We are grateful to Dr. Felipe E. Zilly for providing recombinant proteins and to Dagmar Diezmann for excellent technical assistance and for providing recombinant proteins.

Correspondence should be addressed to Esther Nachliel, Laser Laboratory for Fast Reactions in Biology, Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel. E-mail: eti@hplus.tau.ac.il.

T. Lang's present address: Life & Medical Sciences (LIMES)-Institute, Laboratory for Membrane Biochemistry, University of Bonn, Bonn 53115, Germany.

A. Mezer's present address: Department of Psychology, Stanford University, Stanford, CA 94305.

DOI:10.1523/JNEUROSCI.0710-09.2009

Copyright © 2009 Society for Neuroscience 0270-6474/09/2912292-10\$15.00/0

results to a rigorous kinetics analysis aimed at determining the rate constants of the assembly and disassembly reactions, by combining differential rate equation integration with a heuristic genetic algorithm (GA) search in a multidimensional parameter space (Moscovitch et al., 2004). The only model found capable of reconstructing the experimental observations was based on the study of Sieber et al. (2007) who showed the partitioning of syntaxin molecules into two phases: clustered syntaxin molecules and syntaxin molecules that diffuse in the membranal matrix between the rafts. The analysis suggested different reactivity of the SNAREs present in the two phases: in the clusters, the rate of *cis*-SNARE complex formation was substantially lower, whereas that of their disassembly was significantly higher. The model suggests that syntaxin clusters serve as a mechanism for maintaining reactive SNARE proteins by reducing the overall level of *cis*-SNARE complexes at low energy costs.

Materials and Methods

Measurements of *cis*-SNARE complex dynamics on the inner leaflet of the PM. To record the quasi-equilibrium of *cis*-SNARE complexes (the steady-state condition), neuroendocrine PC12 were grown overnight on poly-L-lysine-coated glass coverslips (Bar-On et al., 2008). Coverslips were sonicated to remove the upper parts of the cells, leaving native PM sheets attached to the coverslips (Avery et al., 2000). The membrane sheets were either directly fixed, to establish control values, or incubated for predetermined times (2–60 min) in a disassembly mixture solution (Bar-On et al., 2008) containing 10 nM NSF and 150 nM α SNAP. After the incubation with the disassembly mixture, the membrane sheets were treated with 8 μ M botulinum neurotoxin C1 (BoNT/C1) for 15 min and then washed briefly and fixed for 1 h at room temperature in 4% paraformaldehyde diluted in PBS. The fixed membrane sheets were washed with PBS and NH_4Cl (diluted in PBS), immunostained, and imaged as described previously (Bar-On et al., 2008). The fluorescence intensities were related to the fluorescence of the control samples, and the values are given as mean $\pm$ SEM ($n = 4$ –7 independent experiments). The assembly and disassembly kinetics were published in a previous study (Bar-On et al., 2008).

Modeling the kinetics and the differential rate equations. The reaction mechanism governing the assembly and disassembly of *cis*-SNARE complexes is based on the presence of syntaxin molecules in non-raft and clustered/raft phases and is modeled by 11 differential equations. The equations include equilibrium between syntaxin molecules in the rafts and outside the rafts and non-identical reactivity (accessibility) of the syntaxin molecules located in the rafts. For the sake of algebraic simplicity, the syntaxin molecules in the rafts were defined in either an active monomeric state or a nonreactive dimeric state. The experimental observation that, immediately after PM sheet formation, $\sim 10\%$ of the syntaxin is already in the form of *cis*-SNARE complexes (Lang et al., 2002; Bar-On et al., 2008) was implemented in the model as well (see Fig. 2) ($t = 0$). For the sake of simplicity, the formation of the *cis*-SNARE- α SNAP-NSF complex and the ATP-driven disassembly reaction were coupled in a single step.

The reactions embodied in the reaction mechanisms corresponding to assembly, disassembly, and quasi-equilibrium were converted into a set of coupled nonlinear ordinary differential equations using the standard chemical-kinetics formalism, according to which the velocity of the reaction is the product of the rate constant of the reaction multiplied by the concentrations of the reactants (Moscovitch et al., 2004). The conversion of the equilibria into differential equations was performed automatically using a custom-written program, ODEBuilder (Bosis et al., 2008).

The simulation procedure. The coupled nonlinear ordinary differential equations were subjected to numerical integration using the Runge Kutta methods as embedded in the Matlab program (the ODE23S routine) (Moscovitch et al., 2004; Mezer et al., 2006). The analyses of the assembly and disassembly of the *cis*-SNARE complexes and the quasi-equilibrium between the two processes were run stepwise. First, we solved the dynamics of the assembly and determined the range of variance for each of the

adjustable parameters pertinent to the assembly process. Next, we analyzed the disassembly dynamics and determined the range of variance for the relevant adjustable parameters. Finally, we reconstructed the whole system as measured under steady-state conditions with known concentrations of added cytoplasmic disassembly factors. In the analysis of the steady-state system, the equations for the assembly and disassembly were gathered, and each of the adjustable parameters was allowed to vary within the range determined by the analyses of the assembly and disassembly reactions.

Determination of search range for unknown parameters. Each adjustable parameter in the set of differential equations was allowed to vary within a given range, depending on its nature. The rate constants in the thermodynamically favored direction were allowed to vary from the upper limit set by the Debye–Smoluchowski equation (Gutman and Nachliel, 1997) to a lower limit set to be eight orders of magnitude lower than the upper limit. The rate constants in the reverse direction were related to the forward ones through the equilibrium constants (Moscovitch et al., 2004; Mezer et al., 2006).

The fitness function. The level of similarity between the experimental signals and the numerical solutions can be expressed by a fitness function $[F(t)]$. We defined $F(t)$ to be weighed as the sum of the absolute value of the deviation of the calculated value ($X_t^{\text{calculated}}$) from the experimental one (X_t^{signal}) and normalized it to X_t^{signal} (Moscovitch et al., 2004; Mezer et al., 2006).

The genetic algorithm. The fitness function was optimized using the GA Optimization Toolbox of Matlab. We used the default parameters of the toolbox as described previously (Moscovitch et al., 2004; Mezer et al., 2006). The analysis was performed by running numerous GA search runs, each lasting 1000 generations. The various runs were scored by the final value of the fitness function. The best 20–40 “phenotypes” were selected, and the values of the various adjustable parameters were evaluated, looking for convergence of each of the parameters. The average values of the adjustable parameters or the converged borders for values that did not fully converge are listed in Table 1.

Results

Experimental measurement of *cis*-SNARE complex kinetics

The experiments analyzed in this study had recorded the temporal level of *cis*-SNARE complexes located on the inner leaflet of the PM (Bar-On et al., 2008). Adherent PC12 cells were subjected to gentle ultrasonic pulse to “unroof” them. All soluble proteins present in the cytoplasmic matrix were removed by exposing the inner leaflet of the PM to a bathing solution. This enabled replacement of the cytoplasm with known concentrations of selected proteins, buffers, and factors.

Observations were recorded for three experimental protocols. The experimental data based on two of them appears in Figures 2 and 6 and was presented previously by Bar-On et al. (2008). For the assembly experiment, the PM sheets were incubated for predetermined times in a bathing solution that was free of cytoplasmic disassembly factors, enabling spontaneous accumulation of *cis*-SNARE complexes (for details, see Bar-On et al., 2008). At selected time points, the membrane sheets were treated with BoNT/C1, which cleaves monomeric syntaxin and syntaxin present in binary complexes (syntaxin–SNAP-25) (Lang et al., 2002), whereas syntaxin molecules that are incorporated into *cis*-SNARE complexes are cleavage resistant (Jahn, 2006). The syntaxin molecules in the *cis*-SNARE complexes were then visualized by immunostaining and quantified by fluorescence microscopy. The increase in PM fluorescence over time therefore represented the accumulation of *cis*-SNARE complexes on the PM (Bar-On et al., 2008).

In the experimental protocol for measuring disassembly dynamics, PM sheets were incubated with externally added fluorescently labeled synaptobrevin, which reacted with the endogenous binary SNARE complexes to form fluorescently labeled *cis*-SNARE complexes (Lang et al., 2002). After 45 min of incubation, during which labeled *cis*-SNARE complexes accumulated on the PM, disassembly factors (NSF and α SNAP in the presence of excess ATP) were added, and the disassembly dynamics was

monitored as the decrement in fluorescence intensity. After the enzymatic disassembly, the labeled synaptobrevin molecules were released to the bathing solution, preventing their reincorporation into *cis*-SNARE complexes (Bar-On et al., 2008).

In the current study, the experimental system was extended to monitor the formation of *cis*-SNARE complexes in the presence of the disassembly factors, reflecting their steady-state level. The cells were sonically disrupted, and the accumulation of *cis*-SNARE complexes on the inner leaflet of the PM was followed in time in the presence of the disassembly mixture containing predetermined concentrations of NSF, α SNAP, and ATP. After selected time intervals, the samples were treated with BoNT/C1 and immunostained as described above (see Fig. 8).

The results were analyzed using a detailed kinetics modeling that expressed all the kinetics reactions as a set of biochemical equilibria. We expressed the assembly and disassembly processes as a set of equilibria between proteins, in which each reaction is characterized by the concentrations of the reactants and the appropriate first- or second-order rate constants (the equations are presented in the supplemental material). The analysis, as detailed in Materials and Methods, proceeded stepwise: the assembly and disassembly dynamics were analyzed independently, using the experimental protocols designed to isolate the two processes from each other. The steady-state dynamics was analyzed with the range of variance limitations for the adjustable parameters that had been obtained from the separate analyses of the two processes.

Analysis of the assembly dynamics

The kinetics of *cis*-SNARE complex assembly revealed a distinct two-phase dynamics (Bar-On et al., 2008): within the first few minutes, there was rapid buildup in the level of *cis*-SNARE complexes, which involved $\sim 15\%$ of the total syntaxin and reached a plateau after 6–8 min. The assembly reaction resumed after a short delay but at a slower rate until a steady-state level was established after 60 min, with $\sim 85\%$ of the syntaxin molecules being part of the *cis*-SNARE complexes (see Fig. 2) (Bar-On et al., 2008).

In the first attempt to reconstruct the experimental signal, we assumed a homogeneous distribution of SNARE proteins on the PM and a sequentially ordered reaction pathway as given by rate constants k_1 and k_2 in Figure 1 (Fasshauer and Margittai, 2004). This model failed to reconstruct the experimental observation: the signal curves generated by the best-fitting phenotypes consistently diverged from the two-phase kinetics of assembly (Fig. 2, curve A). In an attempt to improve the fitness of the simulations, the reaction mechanism was modified. Based on Pobati et al. (2006), we assumed that the native membrane sheets already contain an initial fraction of binary complexes (syntaxin–SNAP-25 complexes) that can directly proceed to form *cis*-SNARE complexes, skipping the rate-limiting step of binary complex formation. However, addition of the preas-

Figure 1. The two-phase reaction pathway. The reactions associated with the formation and disassembly of *cis*-SNARE complexes on the inner leaflet of the PM. The scheme assumes two phases: one is the raft phase, in which the syntaxin is densely packed, and the second is the membrane areas surrounding the rafts in which syntaxin can diffuse freely. The values of the k_i and k'_i represent the rate constants of the same reaction taking place in the raft and non-raft phases, respectively. All other proteins are present in a single pool and can react with the syntaxin molecules present in both of the phases, but reactions rates can differ. The disassembly reaction (k_4 and k'_4) is an ATP-driven, irreversible catalytic reaction. * indicates SNARE monomers: syntaxin, SNAP-25, and synaptobrevin.

sembled binary complex fraction to the model did not yield an accurate reconstruction of the dynamics (supplemental Fig. 1, curve A, available at www.jneurosci.org as supplemental material). An alternative attempt to improve the fit was based on Fasshauer et al. (1997), who showed that, *in vitro*, some of the syntaxin is present in dead-end 2:1 complexes (syntaxin–syntaxin–SNAP-25) (Fasshauer and Margittai, 2004). This model included an additional reaction of the syntaxin molecules with binary complexes forming 2:1 complexes. This model did not manage as well in accurately reconstructing the experimental bi-phasic kinetics (supplemental Fig. 1, curve B, available at www.jneurosci.org as supplemental material).

A more suitable reconstruction of the experimental results (Fig. 2, curve B) was based on Sieber et al. (2007), who showed that syntaxin molecules on the inner leaflet of the PM are located in two distinct phases: in clusters [also classified as membrane rafts (Lang, 2007)] and as free monomers dispersed in the membrane matrix between the rafts. The nonhomogeneous distribution of syntaxin in the two phases was adapted to the model.

Accordingly, the model accounted for syntaxin in two phases that are in equilibrium (Fig. 1): the first phase represents the syntaxin clusters (85% of the total monomeric syntaxin), hereafter the “raft phase,” and the second phase corresponds to the membranal area between the rafts in which syntaxin is present in a free monomeric state (15% of the total monomeric syntaxin). The rate constants k_0 and k_{-0} account for the transfer of syntaxin molecules between these two phases (Fig. 1). The high density of the syntaxin molecules in the raft phase could cause inherent discrimination among the clustered molecules. Those located on the perimeters can react with the membranal proteins SNAP-25 and synaptobrevin outside the rafts at a different rate than syntaxin molecules located in the center of the cluster that have no access to the outside. The rate constants k_{10} and k_{-10} represent

Figure 2. Reconstruction of the kinetics of *cis*-SNARE complex assembly using a single-phase or two-phase model. The figure depicts a reconstruction of the assembly dynamics by integration of the ordinary differential equations using rate constants selected by the GA search. Curve A, Fitting based on a single-phase model. Curve B, Fitting based on a two-phase model in which the syntaxin in the clusters can aggregate into a less reactive/accessible state. Curve C, The expected dynamics given that all of the syntaxin molecules react at the rates of the non-raft phase. The experimental data are marked by squares with SEs. Inset, Expansion of the first 12 min of the assembly dynamics and fitting curves A and B.

Figure 3. The convergence of the adjustable parameters during a GA analysis of the assembly dynamics. The final values (generation number 1000) assigned by each of the independent GA runs to the stated adjustable parameter are presented in each frame. Parameters were searched for in a range of $1\text{--}10^8 \text{ M}^{-1}\text{s}^{-1}$. **A**, k_1 , formation of binary complexes for the membranal non-raft phase. **B**, k_2 , formation of *cis*-SNARE complexes for the membranal non-raft phase. **C**, k'_1 , raft phase. **D**, k'_2 , raft phase. The solid frame represents the defined range of a parameter that converged during the search. The dashed frame represents the partially defined range of a parameter that did not properly converge.

the rate at which a syntaxin molecule alters its accessibility and diffuses to the perimeters of the rafts (Fig. 1). It is reasonable to assume that molecules within the raft phase are not equally accessible to the reactants located outside the raft. It remains to be determined whether such a mechanism involves molecule migration within the aggregate or molecule appearance at the outer rim

as a result of constant cluster remodeling. We applied the simplest assumption to introduce this parameter into the model and, for the sake of algebraic simplicity, replaced the “inner aggregation reaction” by reversible formation of nonreactive dimers of syntaxin molecules. This introduces a reservoir of syntaxin molecules that is not readily available for *cis*-SNARE complex formation.

The analysis of the assembly dynamics by GA, using the reaction pathway presented in Figure 1, was repeated 130 times, each generating a combination of rate constants that reconstructs the observed signal. Of the many solutions generated by the search, the best 30 were considered to be well-fitting phenotypes, and their parameters were used to evaluate the solution. Figure 3 depicts the values of four of the adjustable parameters that were searched for during the analysis. The parameters that converged to a defined range represented those rate constants that were crucial for reconstruction of the dynamics, whereas the non-rate-limiting steps made only a minor contribution to the dynamics and consequently failed to converge. The best-converged parameters in the assembly kinetics were the rate constants for formation of the binary and *cis*-SNARE complexes in the membranal non-raft phase (Fig. 3A,B) and of the *cis*-SNARE complex in the

raft phase (Fig. 3D). A parameter that only partially converged was the rate constant for binary complex formation in the raft phase (k'_1), which appeared to require only a lower limit. Apparently, the experimental data imposed no upper limit on the rate of this reaction; values above $2.9\text{E}3 \text{ M}^{-1}\text{s}^{-1}$ were satisfactory for an accurate reconstruction of the observed dynamics (Fig. 3C).

The analysis indicated that the rate of *cis*-SNARE complex formation in the membranal interspace between the rafts is at least four times faster than the rate in the raft phase (Fig. 3B,D). This strongly indicates that the physicochemical properties characterizing the raft phase differ markedly from those of the membranal matrix between the rafts. Curve C in Figure 2 depicts the dynamics of a hypothetical situation in which all the syntaxin molecules are located in the non-raft phase and exhibit the rates of binary and *cis*-complex formation that were found for this phase.

Most of the syntaxin molecules located in the rafts are incapable of forming binary and *cis*-SNARE complexes, probably attributable to their inaccessibility to the other membranal protein (SNAP-25 or synaptobrevin). Thus, most of the syntaxin molecules in the raft function as a reservoir, keeping syntaxin from entering into *cis*-SNARE complexes. It must be stressed that the model did not make any assumptions regarding the initial ratio of

“reactive-to-inaccessible” syntaxin in the clusters or the rate of the reactivity alternation process of the syntaxin molecules. However, a GA search led to a well-defined range for both parameters, suggesting that at least 90% of the syntaxin present in the clusters is not available for *cis*-SNARE complex formation, possibly because it is embedded in the clusters. The rate constant representing

the accessibility alternation of syntaxin molecules in the clusters (k_{-10}) was on the order of several minutes ($\tau = \sim 6$ min). The time constant for the flux of monomeric syntaxin between the membranal non-raft phase and the raft phase (k_0 and k_{-0}) was on the order of minutes, suggesting that, under the experimental conditions, the syntaxin molecules involved in *cis*-SNARE complex formation located in the rafts are in a very slow equilibrium with those that are located in the membrane matrix between the rafts.

Reconstruction of the *cis*-SNARE complex assembly kinetics by numerical integration of the differential rate equations generated not only the overall dynamics but also the temporal concentrations of all intermediate states, as documented in Figure 4. Frame A depicts *cis*-SNARE complex formation in the non-raft phase: during initiation of the reaction, the concentration of free syntaxin molecules (Fig. 4A, curve a) decreases monotonically, whereas binary complexes (Fig. 4A, curve b) are rapidly formed and are consumed by the formation of *cis*-SNARE complexes (Fig. 4A, curve c). The process is rapid, reaching completion within the first 2–4 min. The fast progression is in accordance with the rate constants of binary and *cis*-SNARE complex formation shown in Figure 3. In the raft phase, the reciprocal relations between the components of the kinetics are more complex, as evident from Figure 4B. Immediately after cytoplasmic matrix elimination, the system approaches equilibrium: the inaccessible syntaxin molecules in the cores of the clusters become accessible for interactions (Fig. 4B, curve d) and engage in *cis*-SNARE complexes (Fig. 4B, curve a). These molecules react with SNAP-25 molecules to form the binary complexes, followed by a reaction with synaptobrevin to form the *cis*-SNARE complexes (Fig. 4B, curves b and c, respectively).

In summary, the analysis suggested that syntaxin is distributed in two phases: a small fraction of the protein (15%), located in the membrane between the clusters, is reactive and rapidly forms *cis*-SNARE complexes. The rest of the syntaxin molecules are stored in clusters in two states of reactivity: the small fraction, 10% or less, is reactive. It is probably situated at the periphery of the clusters in which it can react with SNAP-25 and synaptobrevin molecules. Most of the molecules are not accessible unless they reach the perimeters of the clusters, a process with a time constant of a few minutes. However, the syntaxin molecules at the perimeters of the rafts, although accessible, react at least four times more slowly than the free molecules outside the rafts, suggesting that the raft imposes additional restrictions on the protein–protein interactions.

To verify our projection that the presence of syntaxin in clusters slows down *cis*-SNARE complex assembly, syntaxin clusters were dispersed by cholesterol extraction from the membranes (50 mM methyl- β -cyclodextrin, 30 or 60 min at 4°C), and the assembly kinetics of the *cis*-SNARE complex was measured (at 37°C). Indeed, the measurements showed an acceleration of the assembly kinetics. However, additional kinetic quantitative analysis was not performed because of the experimental limitations of the cholesterol extraction procedure, variability in the effect of the depletion treatment (Lang et al., 2001), and severe structural damages caused to the membranes (data not shown).

Figure 4. Components of *cis*-SNARE complex assembly kinetics. The figure represents the kinetics components of the assembly process in both of the phases as depicted from a representative solution reconstructing the assembly measurement (the corresponding fitting curve is presented in Fig. 2, curve B). **A**, The kinetics of the components of the non-raft phase (during 400 s, until the completion of the dynamics of this phase). **B**, The kinetics of the raft-phase components. Reactive syntaxin (a), binary complexes (b), *cis*-SNARE complexes (c), and nonreactive syntaxin (d).

Figure 5. Syntaxin- α SNAP complex. Reaction added to the disassembly process on top of the basic reaction mechanism presented in Figure 1. The monomeric syntaxin molecules react with α SNAP, and the complex is disassembled by NSF. The rates of these reactions were treated as adjustable parameters (k_5/k_{-5} and k_6 , respectively) (Barszczewski et al., 2008).

Analysis of the disassembly dynamics

The ternary SNARE complex is extremely stable and its spontaneous dissociation rate is very slow, necessitating a specific mechanism: an ATP-driven reaction mediated by α SNAP and NSF (Jahn and Südhof, 1999). The model of the disassembly process is initiated by the binding of α SNAP molecules to a *cis*-SNARE complex, forming the *cis*-SNARE- α SNAP intermediate complex (Fig. 1, k_3). In the next step, NSF binds to the intermediate complex (Fig. 1, k_4) and, in the presence of ATP, degrades the complex back to its components: syntaxin, SNAP-25, synaptobrevin, and α SNAP molecules (Söllner et al., 1993; Hayashi et al., 1995).

In accordance with the experimental procedure that precluded reincorporation of the labeled synaptobrevin (Bar-On et al., 2008) and based on the study of Lauer et al. (2006), the model did not allow recycling of the disassembly products (Fig. 1). Based on the observations of Hanson et al. (1995) and Barszczewski et al. (2008), α SNAP interacts with syntaxin molecules, and this complex is disassembled by NSF. These two reactions were included in the disassembly dynamics model, as detailed in Figure 5.

To be consistent with the results of the assembly experiment, the initial level of *cis*-SNARE complexes (indicating the level of

Figure 6. Two-phase model for the disassembly kinetics. A representative fit reconstructed for the disassembly measurements based on the two-phase model (Fig. 1). The measurements differ in the initial concentrations of NSF and α SNAP. The seven frames represent the kinetics in the presence of $2 \mu\text{M}$ α SNAP and the indicated NSF concentrations (**A–D**) and 40 nM NSF and the indicated α SNAP concentrations (**E–G**) (Bar-On et al., 2008). The presented fit was reconstructed using, as adjustable parameters the reactions k_3 , k_4 and k'_3 , k'_4 , which are presented in Figure 1 and k_5 , k_{-5} , and k_6 in Figure 5.

complexes at $t = 45$ min) (Fig. 2) was set to 70% of the syntaxin content on the PM.

The disassembly kinetics were first analyzed under the assumption that all *cis*-SNARE complexes present on the inner leaflet of the PM are equally available to interact with the disassembly factors added to the bathing solution, being members of a single homogeneous population (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). However, all attempts to reconstruct the dynamics based on a homogeneous population of *cis*-SNARE complexes failed (see an example in supplemental Fig. 2, available at www.jneurosci.org as supplemental material), implying that the *cis*-SNARE complexes generated in the raft phase markedly differ in their susceptibility to the disassembly components from those located in the membranal matrix between the rafts.

The disassembly kinetics were successfully reconstructed by introducing different rate constants for the disassembly process in the non-raft membranal and raft phases (Fig. 1, k_3 , k_4 and k'_3 , k'_4). The GA search treated the values of the disassembly parameters as adjustable parameters that could vary as described in the method section. Repeated GA runs generated many reconstructed dynamics that were compatible with the experimental data, as exemplified in Figure 6. The 29 best-fitting phenotypes were pooled together, and the values of the various adjustable parameters were evaluated.

Figure 7 presents four of the rate constant parameters (k_3 , k_4 , k'_3 , k'_4) used for the analysis of the disassembly dynamics that converged nicely during the reconstruction of the dynamics. The process of α SNAP binding to the *cis*-SNARE complex appeared to be more crucial in the raft phase than in the non-raft membranal phase, as evaluated by the better convergence of the rate constant of the raft phase (Fig. 7, bottom left). The rates of disassembly of the *cis*-SNARE- α SNAP complex by NSF exhibited very consistent values in both phases (Fig. 7, right). Interestingly, disassembly of the complexes in the raft was at least 20-fold faster than in the membranal non-raft matrix. The temporal concentrations of all intermediate states in the disassembly process are presented in supplemental Figure 3 (available at www.jneurosci.org as supplemental material).

Analysis of the steady-state dynamics

In the above sections, we analyzed the dynamics of assembly and disassembly as proceeding independently of one another, by selecting experimental conditions that allowed for only one mechanism to be operational. For a system operating under kinetics control, the two sets of parameters, each corresponding to one pathway (assembly or disassembly), should be sufficient to reconstruct the steady state of the system, in which both assembly and disassembly are operating. To confirm this projection, we performed steady-state measurements and let the GA search for a set of parameters that would reconstruct

the observations, with the strict restriction that the adjustable parameters must be within the range of variance determined by the analysis of both the “isolated” assembly and disassembly pathways. The first time-point measurement of the dynamics, at $t = 2$ min, indicated that only $\sim 8.5\%$ of the syntaxin molecules were in the form of *cis*-SNARE complexes (Fig. 8) compared with 22% of the complexes in the absence of disassembly factors (Fig. 2, $t = 2$ min). This level reflects only the basal *cis*-SNARE complex content of the cell (Lang et al., 2002) and does not include additional spontaneous accumulation of complexes (Fig. 8) during the 2 min as exhibited in the assembly experiment (Fig. 2, $t = 2$ min). With time, the *cis*-SNARE content increased, reaching a steady-state (or quasi-equilibrium) level of 35–40% of the total syntaxin (Fig. 8). This steady-state level was significantly lower than that reached in the absence of disassembly factors (Fig. 2, $t = 60$ min), reflecting the function of the disassembly factors added to the bathing solution. Note that the selected concentrations of α SNAP and NSF that were used in this experimental setup were based on the experiments detailed in Figure 6, so as to reach a steady-state level that would fall between the upper and lower values of the NSF and α SNAP concentrations examined (Figs. 2, 6). Considering that, in freshly prepared PM sheets, only $\sim 10\%$ of the syntaxin is in the *cis*-SNARE-complex form (Lang et al., 2002; Bar-On et al., 2008) implies that the *in vivo* activity or

Figure 7. The convergence of the adjustable parameters during a genetic algorithm analysis of the disassembly dynamics. Each frame depicts the dispersion of the final values (generation number 1000) assigned by each of the independent GA runs ($n = 29$) to the stated adjustable parameter. Parameters are searched for in a range of $1\text{--}10^8 \text{ M}^{-1} \text{ s}^{-1}$. Top, Left, k_3 , formation of SNARE- α SNAP complexes within the non-raft membranal phase. Bottom, Left, k'_3 , raft phase. Top, Right, k_4 , catalytic disassembly of SNARE- α SNAP complexes within the non-raft membranal phase. Bottom, Right, k'_4 (raft phase).

Figure 8. Quasi-equilibrium between assembly and disassembly of *cis*-SNARE complexes forming a new steady-state level of *cis*-SNARE complexes. Black squares, The experimental results of SNARE complex formation in the presence of the disassembly mixture that contains 10 nM NSF and 150 nM α -SNAP (see Materials and Methods). Values are given as mean $\pm$ SEM ($n = 4\text{--}7$ independent experiments). The curve is an example of an accurate reconstruction of the dynamics based on a parameter search performed by genetic algorithm.

concentration of the disassembly factors might be higher than those provided by the experimental conditions selected for this experiment.

The results of the adjustable parameters search are presented in Table 1 and plotted in Figure 9. The analysis, together with the GA search, was repeated 80 times, and Table 1 and Figure 9 include only the convergence of 20 combinations of parameters that yielded the best-fitting phenotypes, i.e., those that most accurately retraced the dynamics as presented in Figure 8. Inspection of the results revealed that some of the parameters converged into a narrow range, and statistical analysis of their distribution (Moscovitch et al., 2004) corresponded with a single normal population. These parameters are marked in Table 1 by their average value and SDs. The reaction mechanism implies that the best-converging parameters are those that control the ob-

Table 1. Compilation of the adjustable parameters needed to reconstruct the experimental observations

Parameter	Results range		Units
	k (non-raft phase)	k' (raft phase)	
k_1	$4.4\text{E}4 \pm 198$	$4\text{E}3\text{--}4.6\text{E}6$	$\text{M}^{-1} \text{s}^{-1}$
k_2	$9.0\text{E}4 \pm 359$	$1.3\text{E}4 \pm 6.4 \text{E}3$	$\text{M}^{-1} \text{s}^{-1}$
k_3	$6.1\text{E}6 \pm 8.3\text{E}5$	$3.2\text{E}5\text{--}4.3\text{E}6$	$\text{M}^{-1} \text{s}^{-1}$
k_4	$1.2\text{E}5 \pm 826$	$2.3\text{E}6\text{--}5.6\text{E}6$	$\text{M}^{-1} \text{s}^{-1}$
τ_{-10}	NA	6.2 ± 3.7	Min
Initial percentage of reactive/accessible syntaxin in raft phase	NA	$\leq 5\%$	%
k (both phases)			
k_5	$3\text{E}5\text{--}1\text{E}7$		$\text{M}^{-1} \text{s}^{-1}$
k_6	$350\text{--}6500$		$\text{M}^{-1} \text{s}^{-1}$

The best-converging parameters are given by their mean $\pm$ SDs. The parameters that converged within boundaries are given by the lower and upper limits. The rate constants for the bimolecular reactions are given in $\text{M}^{-1} \text{s}^{-1}$ and the first-order reactions by the time constant (τ) values.

served dynamics. Other parameters converged only within limits. Although these parameters are essential for the reconstruction of the dynamics, the experimental conditions were such that their effect was rather marginal and the reconstruction was feasible when the given parameters varied within a range. A new experimental design might be required to obtain better resolution of these parameters. Parameters that converged within boundaries are marked in Table 1 by their upper and lower boundaries.

In the membranal non-raft phase, the rate constants associated with the assembly and disassembly converged nicely (Table 1, $k_1\text{--}k_4$; Fig. 9, frames A–D). Such high accuracy is consistent with a reaction in which all reactants are available to each other. In the raft phase, the reaction pathway is more complex because most of the syntaxin molecules are inactive/inaccessible to the other SNARE proteins. This fraction must first become reactive or accessible before it can participate in *cis*-SNARE complex formation. As shown in Table 1, the initial fraction of reactive syntaxin in the rafts was 5% or less, rendering the accessibility process, with a time constant of ~ 6 min, the limiting step for *cis*-SNARE complex formation in the raft phase. The delay in the appearance of reactive syntaxin in the raft phase accounts for the distinct two-phase dynamics observed in the assembly experiments (Fig. 2). Comparison of the rate constants at which *cis*-SNARE complexes are generated in the two phases indicated that, in the raft phase, the reaction of accessible molecules is still approximately eight times slower than in the membranal non-raft phase (Table 1, k'_2 and k_2), probably reflecting a marked difference in the physicochemical properties of the two phases.

The catalytic disassembly of the *cis*-SNARE complexes proceeded simultaneously in the two phases but not at the same velocity. In the non-raft membranal phase, the reaction of *cis*-SNARE complexes with α SNAP (Table 1, k_{-3}) was rapid, and the rate-limiting step was the ATP-driven enzymatic reaction (k_4), which is $\sim 10\%$ of the rate of *cis*-SNARE- α SNAP complex formation. In the raft phase, the rate of binding of α SNAP to the *cis*-SNARE complex was comparable with that in the non-raft phase (k_3 and k'_3), but the following catalytic step was ~ 30 times faster (Fig. 9, frames i and d). A possible biological interpretation of the difference in the catalytic reactions of the two phases is that the higher density of the *cis*-SNARE complexes in the area of the rafts might increase the probability of NSF encountering a *cis*-SNARE- α SNAP complex after disassembly, assuming that NSF stays in the area of the raft.

As shown by Hanson et al. (1995) and Barszczewski et al. (2008), α SNAP can react with individual molecules of syntaxin to

Figure 9. The convergence of the adjustable parameters in the analysis of the steady-state dynamics. The time constant values of the 20 best runs are organized in descending order by fitness function values (frame n). Each frame depicts the dispersion of the final values (generation number 1000) assigned by the search technique to the stated adjustable parameter according to Figures 1 and 2. Parameters were searched in a range of $1\text{--}10^8 \text{ M}^{-1} \text{ s}^{-1}$.

form a stable complex that calls for catalytic dissociation by NSF. As shown in Figure 9, frames J and K, the rate constants of these reactions only partially converged within a range of approximately three orders of magnitude. Apparently, under the experimental setup used here, these reactions had only a marginal influence on the overall dynamics.

The analysis of the experimental data was performed under no assumptions for the rate at which the free syntaxin molecules can be transferred between the non-raft membranal phase and the raft phase. The reconstructions of the experimental measurements consistently indicated that these rate constants fail to converge, suggesting that this process does not affect the observed dynamics (Fig. 9, frames L and M). Moreover, the values of these time constants were on the order of ~ 15 min or longer. Such slow rates, together with the low concentration of free syntaxin, lead to a negligible flux of *cis*-SNARE complex-forming syntaxin molecules between the non-raft membranal and raft phases. Apparently, the syntaxin molecules involved in *cis*-SNARE complex formation located in the membranal phase and those located in the raft phase barely cross the raft boundaries and the syntaxin molecules in the two phases interact with SNAP-25, synaptobrevin, and the disassembly factors with minimal coupling between the phases.

Discussion

This study presents a rigorous analysis of a comprehensive set of measurements consisting of time-resolved quantitative determinations of the formation, disassembly and steady-state dynamics of *cis*-SNARE complexes on the exposed inner leaflet of the PM of PC12 cells. The experimental results were reconstructed through numerical integration of differential rate equations derived from a projected reaction mechanism, in which the rate constants and some of the reactant concentrations were defined as adjustable parameters. The search for a combination of parameters that would reconstruct the experimental observation was performed through heuristic application of a GA. The reconstruction of the results clearly necessitated partitioning of the syntaxin molecules into two phases. It has been reported previously that syntaxin forms cholesterol-dependent clusters that represent docking and fusion sites for secretory granules on the PM (Lang et al., 2001). The syntaxin clusters are immobile (Ohara-Imaizumi et al., 2004; Sieber et al., 2007) and are formed by a homo-oligomerization process through the SNARE motif (Sieber et al., 2006). Disintegration of the clusters by cholesterol extraction inhibits exocytosis, indicating that the clusters are important for this process. However, so far, no mechanistic model explaining the function of

the syntaxin clusters has been found. The syntaxin cluster contains densely crowded syntaxin molecules that are exchanged with a small pool of freely diffusing molecules, the latter consisting of only ~15% of the total syntaxin content on the membrane (Sieber et al., 2007). Interestingly, the kinetics analysis yielded effectively the same ratio between the syntaxin in the two phases, strongly indicating that the biological counterparts of the mathematically defined phases described in this study correspond to the non-raft membranal and raft phases.

Structural considerations suggest that syntaxin clustering in the raft decreases the number of syntaxin molecules available for entering *cis*-SNARE complexes, because most of them are embedded in the center of the clusters. This argument is supported by the kinetics analysis, which suggested that most of the syntaxin in the cluster is not reactive. The high convergence of the level of nonreactive syntaxin in the raft to a consistent value of ~95% (i.e., only 5% of the syntaxin in the raft is accessible/reactive) (Table 1) is an indication of the significance of the inaccessibility of the molecules in the rafts to the reconstruction of the overall dynamics.

The assembly dynamics

After initiation of the assembly reaction, the SNARE proteins on the membrane react with each other, but, because of the non-identical environments, the reaction proceeds at different rates. The non-raft molecules are freer to react, and binary complexes and the ternary *cis*-SNARE complexes are formed within a few minutes. The syntaxin molecules in the rafts can react with the other SNARE proteins only at the perimeters of the rafts and at a lower rate (Table 1). As a result, appearance of the second phase of *cis*-SNARE complex formation is limited by the reaction at the perimeter of the raft and, most importantly, by the time it takes for molecules located in the center of the raft to diffuse to the perimeter and replace the syntaxin molecules that have already reacted, a process that has a time constant of ~6 min.

Disassembly of the *cis*-SNARE complexes

The kinetics analysis indicated that the rates of the catalytic reaction differ substantially between the two phases. The *cis*-SNARE complexes in the raft phase are disassembled ~30 times faster than those in the non-raft phase. A possible explanation for this significant difference is that the *cis*-SNARE complexes formed in the raft phase accumulate in the area, and, therefore, the density of the complexes in the raft area is much higher than outside the rafts. Consequently, once NSF samples the area of the rafts that contains a high local concentration of *cis*-SNARE complexes, it can simply “jump” from complex to complex. The probability of NSF binding a new complex in the packed area of the rafts is higher than outside them, and hence the probability for disassembly is higher as well. It should be stressed that additional experiments are needed to determine the distribution of *cis*-SNARE complexes on the PM when they spontaneously accumulate and to characterize the way in which NSF operates.

Regulation of SNARE reactivity by syntaxin clusters

The ternary SNARE complexes represent the core machinery for membrane fusion (Jahn et al., 2003). However, it is still unclear how their level is regulated. A few regulatory proteins (e.g., Munc18, complexin) have been identified as key participants in the molecular pathway toward vesicle priming and fusion (Malsam et al., 2008). In addition, there is evidence that the overall level of

reactive SNARE complexes determines, if not regulates, exocytotic activity. For instance, in the absence of cytosol, the *cis*-SNARE complexes accumulate, and consequently exocytosis is rapidly inhibited by depriming and requires NSF activity for repriming (Lang et al., 2002).

Considering that reactive SNARE proteins can spontaneously assemble into *cis*-SNARE complexes, there are at least two possible mechanisms regulating their level: acceleration of their disassembly process or a decrease in the rate of their assembly. In the current study, we suggest a dual significant physiological role for the syntaxin clusters. The presence of clusters slows down the formation of non-fusogenic *cis*-SNARE complexes by having most of the syntaxin embedded in the clusters and inaccessible for *cis*-SNARE formation. Furthermore, even syntaxin at the periphery of the clusters is still limited in its ability to react with the SNARE partners, and therefore *cis*-SNARE complex assembly is substantially slower. Concentrating the *cis*-SNARE complexes in defined areas increases the probability of NSF encountering adjacent complexes after complex disassembly in the raft area.

References

- Avery J, Ellis DJ, Lang T, Holroyd P, Riedel D, Henderson RM, Edwardson JM, Jahn R (2000) A cell-free system for regulated exocytosis in PC12 cells. *J Cell Biol* 148:317–324.
- Bar-On D, Winter U, Nachliel E, Gutman M, Fasshauer D, Lang T, Ashery U (2008) Imaging the assembly and disassembly kinetics of *cis*-SNARE complexes on native plasma membranes. *FEBS Lett* 582:3563–3568.
- Barszczewski M, Chua JJ, Stein A, Winter U, Heintzmann R, Zilly FE, Fasshauer D, Lang T, Jahn R (2008) A novel site of action for α -SNAP in the SNARE conformational cycle controlling membrane fusion. *Mol Biol Cell* 19:776–784.
- Bosis E, Nachliel E, Cohen T, Takeda Y, Ito Y, Bar-Nun S, Gutman M (2008) Endoplasmic reticulum glucosidase II is inhibited by its end products. *Biochemistry* 47:10970–10980.
- Fasshauer D (2003) Structural insights into the SNARE mechanism. *Biochim Biophys Acta* 1641:87–97.
- Fasshauer D, Margittai M (2004) A transient N-terminal interaction of SNAP-25 and syntaxin nucleates SNARE assembly. *J Biol Chem* 279:7613–7621.
- Fasshauer D, Otto H, Eliason WK, Jahn R, Brünger AT (1997) Structural changes are associated with soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor complex formation. *J Biol Chem* 272:28036–28041.
- Gutman M, Nachliel E (1997) Time-resolved dynamics of proton transfer in proteinous systems. *Annu Rev Phys Chem* 48:329–356.
- Hanson PI, Otto H, Barton N, Jahn R (1995) The N-ethylmaleimide-sensitive fusion protein and α -SNAP induce a conformational change in syntaxin. *J Biol Chem* 270:16955–16961.
- Hayashi T, Yamasaki S, Nauenburg S, Binz T, Niemann H (1995) Disassembly of the reconstituted synaptic vesicle membrane fusion complex in vitro. *EMBO J* 14:2317–2325.
- Jahn R (2006) Neuroscience. A neuronal receptor for botulinum toxin. *Science* 312:540–541.
- Jahn R, Südhof TC (1999) Membrane fusion and exocytosis. *Annu Rev Biochem* 68:863–911.
- Jahn R, Lang T, Südhof TC (2003) Membrane fusion. *Cell* 112:519–533.
- Lang T (2003) Imaging SNAREs at work in “unroofed” cells: approaches that may be of general interest for functional studies on membrane proteins. *Biochem Soc Trans* 31:861–864.
- Lang T (2007) SNARE proteins and “membrane rafts.” *J Physiol* 585:693–698.
- Lang T, Bruns D, Wenzel D, Riedel D, Holroyd P, Thiele C, Jahn R (2001) SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis. *EMBO J* 20:2202–2213.
- Lang T, Margittai M, Hölzler H, Jahn R (2002) SNAREs in native plasma membranes are active and readily form core complexes with endogenous and exogenous SNAREs. *J Cell Biol* 158:751–760.
- Lauer JM, Dalal S, Marz KE, Nonet ML, Hanson PI (2006) SNARE complex zero layer residues are not critical for N-ethylmaleimide-sensitive factor-mediated disassembly. *J Biol Chem* 281:14823–14832.

- Littleton JT, Barnard RJ, Titus SA, Slind J, Chapman ER, Ganetzky B (2001) SNARE-complex disassembly by NSF follows synaptic-vesicle fusion. *Proc Natl Acad Sci U S A* 98:12233–12238.
- Malsam J, Kreye S, Söllner TH (2008) Membrane fusion: SNAREs and regulation. *Cell Mol Life Sci* 65:2814–2832.
- Mezer A, Ashery U, Gutman M, Project E, Bosis E, Fibich G, Nachliel E (2006) Systematic search for the rate constants that control the exocytotic process from chromaffin cells by a genetic algorithm. *Biochim Biophys Acta* 1763:345–355.
- Moscovitch D, Noivirt O, Mezer A, Nachliel E, Mark T, Gutman M, Fibich G (2004) Determination of a unique solution to parallel proton transfer reactions using the genetic algorithm. *Biophys J* 87:47–57.
- Ohara-Imaizumi M, Nishiwaki C, Nakamichi Y, Kikuta T, Nagai S, Nagamatsu S (2004) Correlation of syntaxin-1 and SNAP-25 clusters with docking and fusion of insulin granules analysed by total internal reflection fluorescence microscopy. *Diabetologia* 47:2200–2207.
- Otto H, Hanson PI, Jahn R (1997) Assembly and disassembly of a ternary complex of synaptobrevin, syntaxin, and SNAP-25 in the membrane of synaptic vesicles. *Proc Natl Acad Sci U S A* 94:6197–6201.
- Pobbati AV, Stein A, Fasshauer D (2006) N- to C-terminal SNARE complex assembly promotes rapid membrane fusion. *Science* 313:673–676.
- Sanyal S, Tolar LA, Pallanck L, Krishnan KS (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.
- Siebert JJ, Willig KI, Heintzmann R, Hell SW, Lang T (2006) The SNARE motif is essential for the formation of syntaxin clusters in the plasma membrane. *Biophys J* 90:2843–2851.
- Siebert JJ, Willig KI, Kutzner C, Gerding-Reimers C, Harke B, Donnert G, Rammner B, Eggeling C, Hell SW, Grubmüller H, Lang T (2007) Anatomy and dynamics of a supramolecular membrane protein cluster. *Science* 317:1072–1076.
- Söllner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE (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.