JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Article
pubs.acs.org/JACS

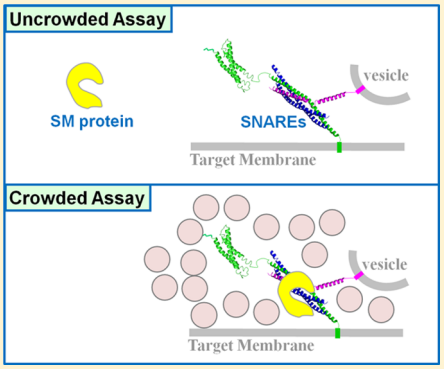
Reconstituting Intracellular Vesicle Fusion Reactions: The Essential Role of Macromolecular Crowding

Haijia Yu,[‡] Shailendra S. Rathore,[†,‡] Chong Shen, Yinghui Liu, Yan Ouyang, Michael H. Stowell, and Jingshi Shen*

Department of Molecular, Cellular and Developmental Biology, University of Colorado at Boulder, Boulder, Colorado 80309, United States

Supporting Information

ABSTRACT: Intracellular vesicle fusion is mediated by SNAREs and Sec1/Munc18 (SM) proteins. Despite intensive efforts, the SNARE-SM mediated vesicle fusion reaction has not been faithfully reconstituted in biochemical assays. Here, we present an unexpected discovery that macromolecular crowding is required for reconstituting the vesicle fusion reaction in vitro. Macromolecular crowding is known to profoundly influence the kinetic and thermodynamic behaviors of macromolecules, but its role in membrane transport processes such as vesicle fusion remains unexplored. We introduced macromolecular crowding agents into reconstituted fusion reactions to mimic the crowded cellular environment. In this crowded assay, SNAREs and SM proteins acted in concert to drive efficient membrane fusion. In uncrowded assays, by contrast, SM proteins failed to associate with the SNAREs and the fusion rate decreased more than 30-fold, close to undetectable levels. The activities of SM proteins were strictly specific to their cognate SNARE isoforms and sensitive to biologically relevant mutations, further supporting that the crowded fusion assay accurately recapitulates the vesicle fusion reaction. Using this crowded fusion assay, we also showed that the SNARE-SM mediated fusion reaction can be modulated by two additional factors: NSF and α-SNAP. These findings suggest that the vesicle fusion machinery likely has been evolutionarily selected to function optimally in the crowded milieu of the cell. Accordingly, macromolecular crowding should constitute an integral element of any reconstituted fusion assay.



INTRODUCTION

A large fraction of intracellular volume is occupied by macromolecules such as proteins and RNAs. As a result, the effective concentration of a specific macromolecule is substantially higher than its actual concentration.[1] This macromolecular crowding effect (also known as excluded volume effect) profoundly alters the kinetic and thermodynamic behaviors of macromolecules,[2] and can increase macromolecular association by orders of magnitude.[1b,3] Indeed, biochemical assays reconstituted with macromolecular crowding agents recapitulate cellular processes more faithfully than uncrowded assays.[1] Surprisingly, the role of macromolecular crowding in intracellular vesicle fusion—a widely studied fundamental biological pathway—has not been examined.

In a vesicle fusion reaction, a membrane-bound trafficking vesicle merges with the lipid bilayer of its target organelle, delivering the cargo it carries. All vesicle fusion reactions require a conserved core machinery composed of SNAREs (soluble *N*-ethylmaleimide sensitive factor attachment protein receptors) and SM (Sec1/Munc18) proteins.[4] SNAREs are membrane-tethered proteins that contain characteristic sequences of 60−70 residues known as core domains or SNARE motifs.[5] To drive membrane fusion, the core domains of the vesicle-rooted SNARE (v-SNARE) and the target membrane-associated SNAREs (t-SNAREs) pair and zipper into a four-helix *trans*-SNARE complex between two apposed bilayers.[4a,c,6] N- to C-terminal zippering of the *trans*-SNARE complex (also known as SNAREpin) brings the two membranes into close apposition to fuse.[7] SM proteins, on the other hand, are soluble factors that promote membrane fusion through binding to their cognate SNAREs.[8]

After fusion, the postfusion *cis*-SNARE complex is dissociated into individual subunits by two universal soluble factors—NSF and α-SNAP—using energy derived from ATP hydrolysis.[9] As a result, the SNAREs are regenerated to prepare for the next round of vesicle fusion. Inactivation of NSF and α-SNAP blocks all vesicle fusion events in the cell due to rapid consumption of free v- and t-SNAREs.[9c]

To establish the principles of intracellular vesicle fusion, it is necessary to develop compositionally defined reconstituted assays using purified components. Of the conserved molecules in vesicle fusion, SNAREs, NSF, and α-SNAP have been extensively studied and their molecular mechanisms are now well established.[9a,b,10] The molecular basis of how SM proteins control vesicle fusion, however, has not been fully elucidated. Given the central role of SM proteins in vesicle fusion, a reconstituted fusion assay cannot be considered complete until

Received: May 18, 2015
Published: October 2, 2015

DOI: 10.1021/jacs.5b08306
J. Am. Chem. Soc. 2015, 137, 12873−12883

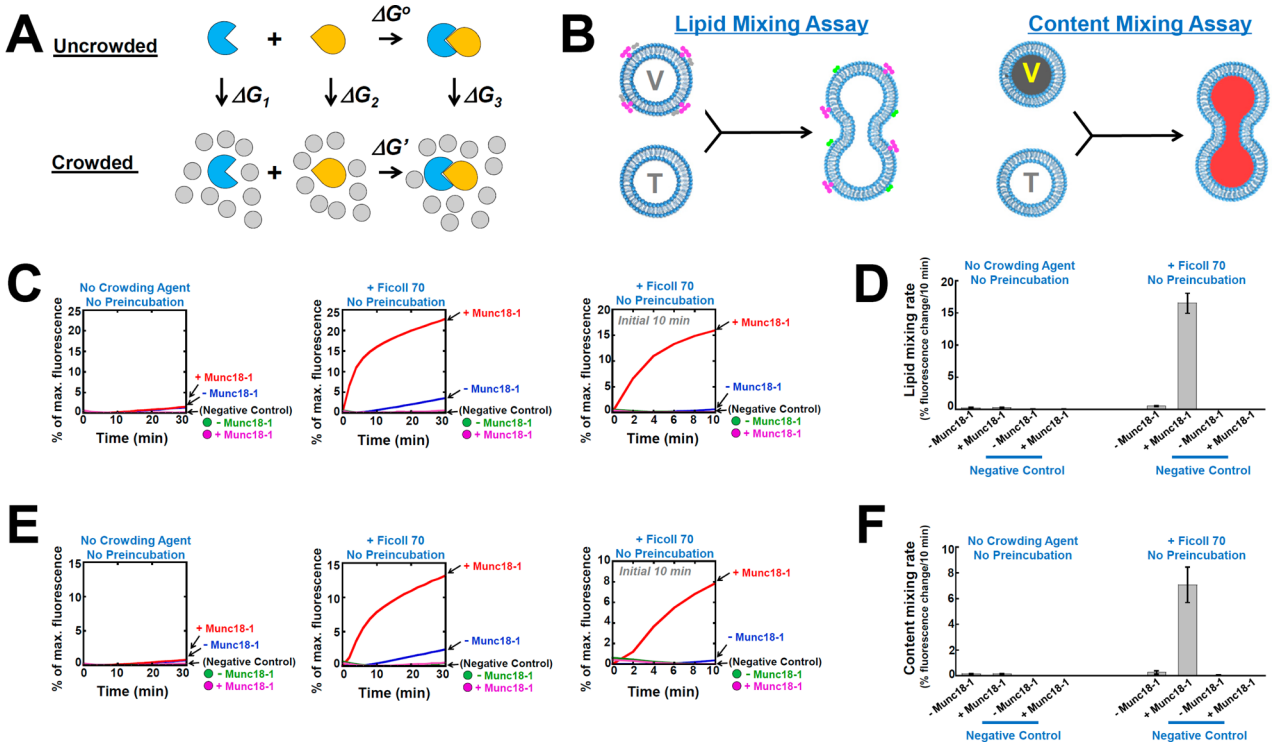


Figure 1. Munc18−1 potently stimulates membrane fusion in the presence of macromolecular crowding agents, without requiring low-temperature preincubation. (A) The free energy landscape of a macromolecular association reaction in crowded and uncrowded environment. The reactions are described as $\Delta G' - \Delta G^\circ = \Delta G_3 - \Delta G_1 - \Delta G_2$, in which $\Delta G'$ and ΔG° denote the free energy changes of the association reactions whereas ΔG_{1-3} denotes the free energy changes associated with the relocation of the corresponding molecule from uncrowded to crowded environment. (B) Diagrams showing the assays that measure the lipid mixing and content mixing of liposome fusion reactions. For clarity, proteins and crowding agents are not shown. (C) The t-SNARE liposomes containing syntaxin-1 and SNAP-25 were directed to fuse with VAMP2 liposomes in the absence or presence of 5 μM Munc18−1. The reactions were carried out with or without 100 mg/mL Ficoll 70. Ingredients of the samples were mixed and immediately loaded into a prewarmed microplate to initiate fusion. The fusion reactions were measured by the FRET-based lipid mixing assay. In negative controls, CDV2 was added to the reactions to the final concentration of 20 μM. The right graph depicts the first 10 min of the fusion reaction shown in the middle graph, aiming to illustrate the stimulatory activity of Munc18−1 in the initial stage of the fusion reaction. (D) Initial lipid-mixing rates of the fusion reactions shown in C. Error bars indicate standard deviation. (E) The fusion reactions were performed as in C and measured by the content mixing assay. The right graph depicts the first 10 min of the fusion reaction shown in the middle graph. (F) Initial content-mixing rates of the fusion reactions shown in E. Error bars indicate standard deviation.

the cognate SM protein is included. SM proteins have been shown to bind different SNARE assemblies and the binding mode appears to vary across species or pathways.[4b,11] It poses significant challenges to define which SNARE-binding mode(s) represents the conserved function of SM proteins in the cell.

Two mammalian SM proteins—Munc18−1 and Munc18c—have attracted substantial attention because of their broad implications in physiology and disease. Munc18−1 (also known as nSec1 or STXBP1) is involved in synaptic exocytosis whereas Munc18c (also known as Munc18−3 or STXBP3) regulates nonsynaptic exocytic pathways.[4b,10−12] In reconstituted assays, both Munc18−1 and Munc18c positively regulate the SNARE-dependent fusion reaction by accelerating the zippering of the *trans*-SNARE complex.[8b,c,11a,13] Moreover, binding to SNARE complexes appears to be a conserved feature of the SM protein family.[14] Thus, the *trans*-SNARE-regulating mechanism revealed in reconstituted assays can potentially explain the essential role of SM proteins in vesicle fusion.

Existing reconstituted assays with SM proteins, however, exhibit two notable limitations. First, the stimulatory effects of SM proteins are often not strong enough to account for the severe knockout phenotypes of SM proteins in vivo.[8b,13,15] Second, a low-temperature (e.g., 4 °C) preincubation step is usually necessary to observe the stimulatory effects of SM proteins.[8b,11a,13] The low-temperature preincubation likely facilitates the association of SM proteins with a metastable SNARE intermediate in vitro but this artificial manipulation is difficult to correlate with physiology. Thus, a fundamentally improved reconstitution system is needed to accurately recapitulate SM protein functions in vesicle fusion.

In this study, we present a surprising finding that macromolecular crowding is crucial to the reconstitution of SM protein functions in vitro. Macromolecular crowding agents were introduced into reconstituted fusion reactions to mimic the crowded intracellular environment (Figure 1A). Strikingly, in this crowded fusion assay, the SM proteins Munc18−1 and Munc18c potently stimulated SNARE-dependent membrane fusion without requiring any low-temperature preincubation. SM proteins increased the initial fusion rate more than 30-fold, consistent with the knockout phenotypes of SM proteins. Thus, inclusion of macromolecular crowding resolves both the potency and preincubation issues associated with uncrowded assays, and recapitulates the in vivo dependency of vesicle fusion on SM proteins. Importantly, the stimulatory activities of SM proteins in these reactions were strictly specific to their cognate SNARE isoforms and sensitive to physiologically relevant SNARE mutations, further supporting that the crowded fusion assay recapitulates the biological function of

DOI: 10.1021/jacs.5b08306
J. Am. Chem. Soc. 2015, 137, 12873−12883

SM proteins. Finally, we demonstrated that NSF and α-SNAP both positively and negatively modulate the SNARE-SM mediated fusion reaction. These findings suggest that the vesicle fusion machinery likely has been evolutionarily selected to optimally drive membrane fusion under the macromolecular crowding condition of the cell.

MATERIALS AND METHODS

Protein Expression and Purification. Recombinant t- and v-SNARE proteins were expressed and purified as we previously described.[13,16] The synaptic exocytic t-SNARE complex was composed of untagged rat syntaxin-1 and mouse SNAP-25 with an N-terminal His_6 tag. The GLUT4 exocytic t-SNARE complex was composed of untagged rat syntaxin-4 and mouse His_6-tagged SNAP-23. Recombinant v-SNARE proteins had no extra residues left after the tags were removed. The v-SNARE mutants were generated by site-directed mutagenesis and purified similarly to WT proteins. SNAREs were stored in a buffer containing 25 mM HEPES (pH 7.4), 400 mM KCl, 1% n-octyl-β-D-glucoside (OG), 10% glycerol, and 0.5 mM Tris(2-carboxyethyl)phosphine (TCEP). Soluble factors were stored in the protein binding buffer (25 mM HEPES [pH 7.4], 150 mM KCl, 10% glycerol, and 0.5 mM TCEP).

Recombinant untagged Munc18−1 and Munc18c proteins were produced in *E. coli* and *Sf9* insect cells, respectively, using procedures we previously established.[8b,11a,15a,17] To preserve their maximum activities, purified SM proteins were immediately frozen, stored at −70 °C, and used within one month of purification. Full-length (FL) rat synaptotagmin-1 was expressed and purified in the similar way as we described for VAMP2. Human complexin-1 was expressed and purified using the protocol of Munc18−1 preparation.

Proteoliposome Reconstitution. All lipids were obtained from Avanti Polar Lipids Inc. For t-SNARE reconstitution, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) and cholesterol were mixed in a molar ratio of 60:20:10:10. For v-SNARE reconstitution, POPC, POPE, POPS, cholesterol, (*N*-(7-nitro-2,1,3-benzoxadiazole-4-yl)-1,2-dipalmitoylphosphatidylethanolamine (NBD-DPPE) and *N*-(Lissamine rhodamine B sulfonyl)-1,2-dipalmitoylphosphatidylethanolamine (rhodamine-DPPE) were mixed at a molar ratio of 60:17:10:10:1.5:1.5. SNARE proteoliposomes were prepared by detergent dilution and isolated on a Nycodenz density gradient flotation.[13] Complete detergent removal was achieved by overnight dialysis of the samples in Novagen dialysis tubes against the reconstitution buffer (25 mM HEPES [pH 7.4], 100 mM KCl, 10% glycerol, and 1 mM DTT). To prepare sulforhodamine-loaded liposomes, t- or v-SNARE liposomes were reconstituted in the presence of 50 mM sulforhodamine B (Sigma). Free sulforhodamine B was removed by overnight dialysis followed by liposome flotation on a Nycodenz gradient. The protein: lipid ratio was at 1:200 for v-SNAREs and at 1:500 for t-SNARE liposomes.

Lipid Mixing and Content Mixing Assays. A standard lipid mixing reaction contained 45 μL of unlabeled t-SNARE liposomes and 5 μL of v-SNARE liposomes labeled with NBD and rhodamine, and was conducted in a 96-well microplate at 37 °C.[18] The NBD-fluorescence (excitation: 460 nm; emission: 538 nm) was measured every 2 min in a BioTek Synergy HT microplate reader. For content mixing assays,[11a,19] unlabeled t-SNARE liposomes were directed to fuse with sulforhodamine B-loaded v-SNARE liposomes. The sulforhodamine B fluorescence (excitation: 565 nm; emission: 585 nm) was measured every 2 min. At the end of the reaction, 10 μL of 10% CHAPSO was added to each sample.

To assess the regulatory activities of SNARE-binding molecules, v- and t-SNARE liposomes were mixed with the regulators and immediately loaded into a preheated microplate (37 °C) to initiate fusion. The fusion reactions were performed as we previously described,[11a,15a] except that no low-temperature preincubation was included in any of the fusion reactions in this study. Fusion data were presented as the percentage of maximum fluorescence change. The maximum fusion rate within the first 10 min of liposome fusion reaction was used to represent the initial rate of a fusion reaction. Full accounting of statistical significance was included for each figure based on at least three independent experiments.

To introduce macromolecular crowding agents, Ficoll 70 (GE Healthcare), bovine serum albumin (BSA, Fisher), and Dextran 70 (Fluka) were separately dissolved in the reconstitution buffer. To remove impurities, BSA was dialyzed overnight in a dialysis bag against the reconstitution buffer with Bio-Beads (Bio-Rad) added. The final concentration of each crowding agent in the reactions was 100 mg/mL. SM proteins promote fusion with such potency that it is critical to start all fusion reactions immediately after mixing (less than 1 min). Otherwise, SM protein-containing liposomes would fuse during the preparation period, yielding inaccurate initial fluorescence readings.

Liposome Coflotation Assay. Liposomes containing the t-SNARE complex (syntaxin-1 and SNAP-25) were incubated with the GST-tagged cytoplasmic domain of VAMP2 (GST-CDV2) for 1 h at 4 °C to assemble the *cis*-SNARE complex. The Mg^{2+}-premix and EDTA-premix were prepared as follows: 12 μL of 1 M creatine phosphate (Roche), 15 μL of 4 mg/mL creatine kinase (Sigma), 5 μL of 0.2 M ATP (Sigma), 2 μL of 0.5 M $MgCl_2$ (or EDTA), 24 μL of 1.8 mg/mL NSF, and 22 μL of 3.7 mg/mL α-SNAP. Liposomes containing *cis*-SNARE complexes were incubated with the Mg^{2+}-premix or EDTA-premix for 1 h at 37 °C. The samples were subsequently loaded onto a Nycodenz gradient and centrifuged at 52 000 rpm for 4 h in a SW55 rotor (Beckman Coulter). Liposomes were collected from the top of the Nycodenz gradient and analyzed by SDS-PAGE.

Neuronal Culture and Lentiviral Infection. Cortical neurons isolated from newborn mice were dissociated by papain (Worthington) digestion and seeded on glass coverslips coated with Poly-D-lysine (Sigma). The cells were grown in the Neurobasal Medium (Life Technologies) supplemented with B-27 (Life Technologies), glutaMAX (Life Technologies), and Ara-C (Sigma). To silence VAMP2 expression, a VAMP2-targeting lentiviral shRNA plasmid (TRCN0000110540, Sigma) was obtained from University of Colorado Functional Genomics Facility. The shRNA targets a 21-bp sequence (5′-CCGACCACAATCTGGTTCTTT-3′) in the 3′ UTR region of the mouse VAMP2 gene. To introduce the rescue VAMP2 gene, the lentiviral shRNA plasmid was digested with *Bam*HI and *Kpn*I to remove the puromycin resistance gene (puroR). Mouse VAMP2 gene was subcloned into the *Bam*HI and *Kpn*I sites of the lentiviral vector. In these constructs, the VAMP2 shRNA was coexpressed with the VAMP2 rescue gene. Knockdown efficiency and rescue gene expression were examined by immunoblotting. Lentiviral particles were produced as we previously described.[20] Neurons were infected with the lentiviruses at DIV 6, and analyzed by electrophysiological measurements at DIV 16−20.

Electrophysiological Recordings. Electrophysiological measurements of synaptic release were performed following a previously established procedure.[21] Evoked synaptic releases were induced by one-millisecond current injections using a concentric bipolar microelectrode (FHC; Model: CBAEC75) placed about 100−150 μm from the cell bodies of patched neurons. The extracellular stimuli were manipulated using an Isolated Pulse Stimulator (World Precision Instruments). The evoked responses were measured by whole-cell recordings using a Multiclamp 700B amplifier (Molecular Devices). The whole-cell pipet solution contained 135 mM CsCl, 10 mM HEPES-CsOH (pH 7.25), 0.5 mM EGTA, 2 mM $MgCl_2$, 0.4 mM NaCl-GTP, and 4 mM NaCl-ATP. The bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.8 $MgCl_2$, 10 mM HEPES-NaOH (pH 7.4), and 10 mM Glucose. The mEPSCs of the neurons were sampled at 10 kHz in the presence of 1 μM tetrodotoxin (TTX, Sigma). The resistance of pipettes was 3−5 mega ohms. The series resistance was adjusted to 8−10 mega ohms once the whole-cell configuration was established. To construct cumulative charge histograms, responses recorded from each neuron were integrated over 0.8 s for individual action potential stimulation. The time constants of evoked responses were calculated using the following equation: $y = y_0 + A_1{*}\exp(-(x - x_0)/t_1) + A_2{*}\exp(-(x - x_0)/t_2)$.

DOI: 10.1021/jacs.5b08306
J. Am. Chem. Soc. 2015, 137, 12873−12883

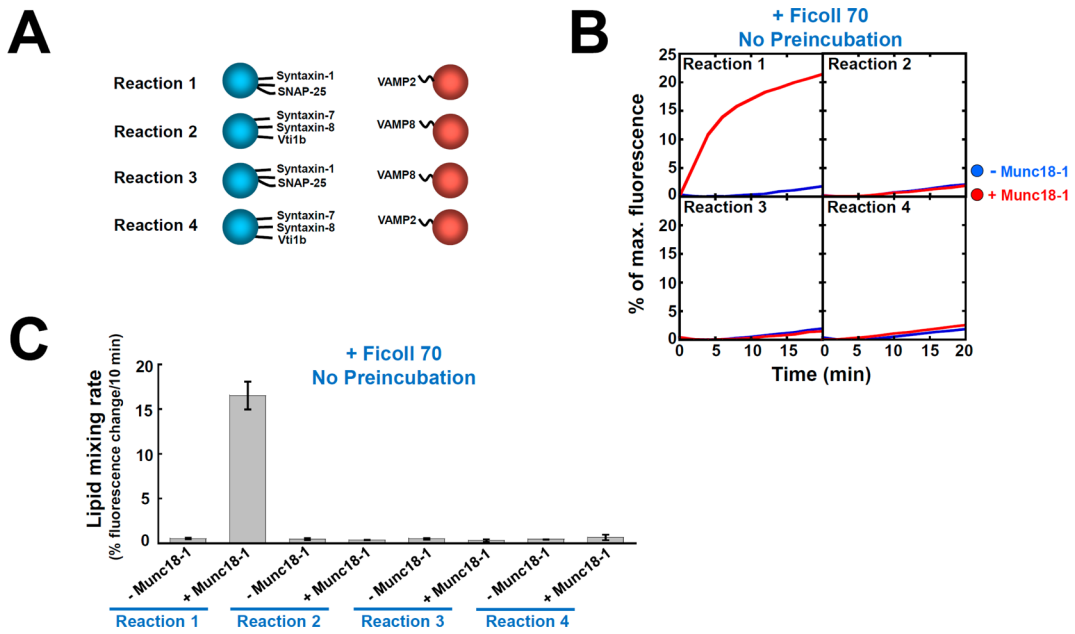


Figure 2. The compartmental specificity of Munc18−1 function is recapitulated in the presence of macromolecular crowding agents. (A) Illustrations of the liposome fusion pairs. (B) Fusion reactions depicted in A were carried out in the absence or presence of 5 μM Munc18−1. All the fusion reactions contained 100 mg/mL Ficoll 70. Ingredients of the samples were mixed and immediately loaded into a prewarmed microplate to initiate fusion. The fusion reactions were measured by the FRET-based lipid mixing assay. (C) Initial lipid-mixing rates of the fusion reactions shown in B. Error bars indicate standard deviation.

The detailed numbers of cultures and neurons are listed in Table S2. The electrophysiological data were analyzed using the pClamp 10 software (Molecular Devices). For statistical calculations, all data are shown as means ± SEMs. The p values were calculated using Student's t-test. In gene rescue experiments, all data are compared to control WT neurons.

RESULTS

The Stimulatory Function of Munc18−1 in Reconstituted Membrane Fusion Reactions Is Dependent on Macromolecular Crowding. We reconstituted the three SNAREs—syntaxin-1, SNAP-25, and VAMP2—into proteoliposomes and performed liposome fusion reactions without any low-temperature preincubation. We first monitored the fusion of v- and t-SNARE liposomes using a fluorescence resonance energy transfer (FRET)-based lipid mixing assay (Figure 1B).[6a] The SNAREs drove a basal level of lipid mixing (Figure 1C,D). As expected, Munc18−1 did not significantly enhance the SNARE-mediated lipid mixing without preincubation with the SNARE liposomes (Figure 1C,D). Next, we carried out the same fusion reactions in the presence of 100 mg/mL Ficoll 70, a widely used macromolecular crowding agent.[3a] The basal SNARE-mediated lipid mixing was only moderately enhanced by Ficoll 70 (Figure 1C,D). Strikingly, we observed that Munc18−1 dramatically stimulated the lipid mixing of the fusion reaction in the presence of Ficoll 70, without requiring low-temperature preincubation (Figure 1C,D). We estimated that the initial lipid mixing rate increased more than 30-fold in the presence of Munc18−1. In fact, in the initial stage (e.g., 10 min) of the reaction, virtually no lipid mixing occurred unless Munc18−1 was added (Figure 1C, right). Addition of the dominant negative inhibitor CDV2 (the cytoplasmic domain of VAMP2) reduced the lipid mixing to background levels (Figure 1C,D).

Similarly, Munc18−1 did not significantly promote the content mixing of the fusion reaction in the absence of Ficoll 70 (Figure 1E,F). When 100 mg/mL Ficoll 70 was included, however, Munc18−1 potently stimulated the content mixing without low-temperature preincubation (Figure 1E,F). No content leakage was observed in any of the Ficoll 70-containing fusion reactions (Figure S1), indicating that bona fide membrane fusion occurred. Addition of Ficoll 70 resulted in a small osmotic concentration gradient (Δosmotic concentration = 14 mOsm/L) across the liposome membrane. To eliminate this gradient, the lumen of the SNARE liposomes was loaded with 100 mg/mL Ficoll 70. We observed that the stimulatory activities of Munc18−1 remained the same in the reactions using Ficoll 70-loaded liposomes (Figure S2).

To preclude the possibility that Ficoll 70 directly binds vesicle fusion proteins, we tested another crowding agent BSA. We observed that, in the presence of 100 mg/mL BSA, Munc18−1 robustly stimulated both the lipid mixing and leakage-free content mixing of the fusion reactions without requiring low-temperature preincubation (Figure S3). Similar results were obtained when the macromolecular crowding agent Dextran 70 was used (Figure S4). By contrast, Munc18−1 failed to stimulate SNARE-dependent fusion reaction in the presence of the small molecule sucrose (Figure S5). The SNARE-dependent fusion reaction is initiated by the pairing of the N-terminal domains of the v- and t-SNAREs, a step known as vesicle docking.[7c] In a liposome docking assay,[11a] we observed that Munc18−1 had little effect on the docking of the SNARE liposomes in the presence of Ficoll 70 (Figure S6). In a *trans*-SNARE formation assay,[11a] Munc18−1 strongly accelerated the zippering of the *trans*-SNARE complex in the presence of Ficoll 70 (Figure S7). Thus, Munc18−1 primarily acts at the postdocking stage of the fusion reaction.

Together, these findings demonstrate that, in the presence of macromolecular crowding agents, Munc18−1 activates SNARE-dependent membrane fusion without requiring low-temperature preincubation. Munc18−1 stimulated membrane

DOI: 10.1021/jacs.5b08306
J. Am. Chem. Soc. 2015, 137, 12873−12883

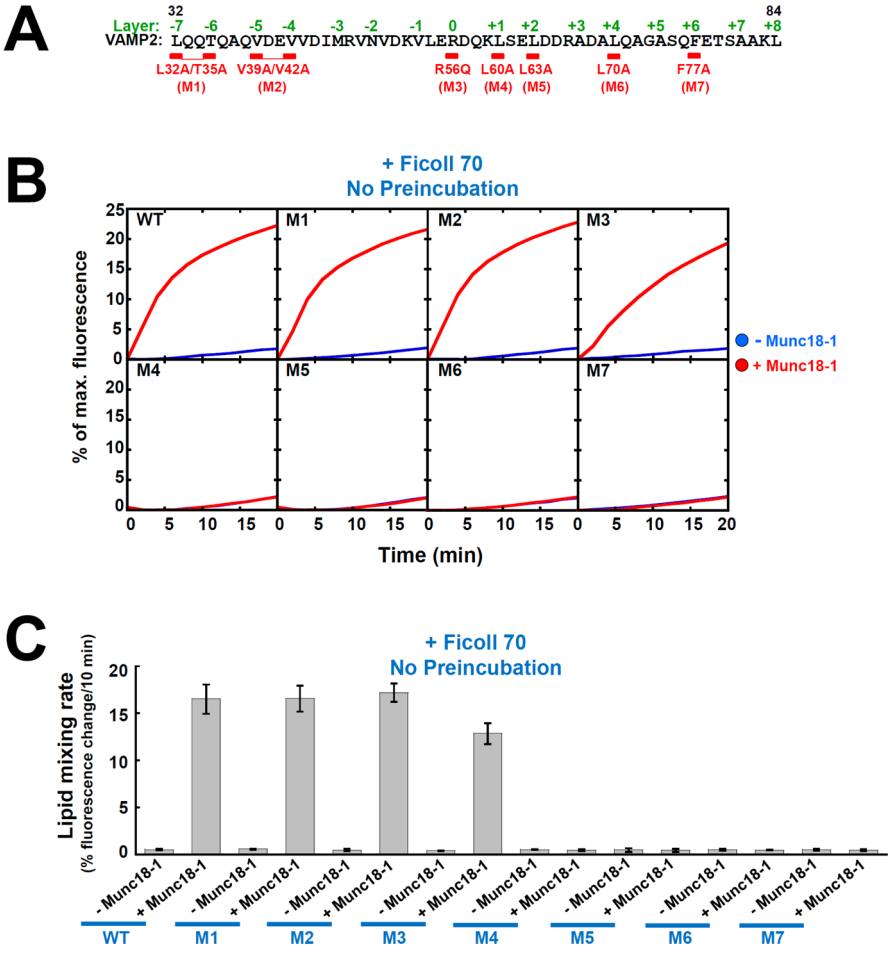


Figure 3. The stimulatory function of Munc18−1 in the crowded fusion assay is sensitive to v-SNARE mutations. (A) The SNARE motif sequence (a.a. 32−84) of VAMP2. Layer residues are marked on the top. Residues mutated in this study are labeled by red bars. (B) Fusion reactions with VAMP2 WT or mutants depicted in A were carried out in the absence or presence of 5 μM Munc18−1. All the reactions contained 100 mg/mL Ficoll 70. Ingredients of the samples were mixed and immediately loaded into a prewarmed microplate to initiate fusion. The fusion reactions were measured by the FRET-based lipid mixing assay. (C) Initial lipid-mixing rates of the fusion reactions shown in B. Error bars indicate standard deviation.

fusion with such potency that the initial stage of the fusion reaction was wholly Munc18−1 dependent (Figure 1C and E, right), correlating with the dependency of vesicle fusion on SM proteins in vivo. Thus, inclusion of macromolecular crowding agents resolved both the potency and preincubation issues associated with previous reconstituted assays of Munc18−1, supporting the hypothesis that the crowded fusion assay recapitulates the function of Munc18−1 in vesicle fusion.

Munc18−1 Selectively Activate Its Cognate SNARE Isoforms in Crowded Fusion Reactions. Next we sought to further examine the biological relevance of the crowded fusion assay. The activities of SM proteins are strictly pathway specific in vivo,[11b] and this compartmental specificity is expected to be recapitulated in reconstituted fusion assays. To test this, we reconstituted the SNAREs involved in lysosomal/late endosomal fusion—syntaxin-7, syntaxin-8, Vti1b, and VAMP8/endobrevin—into proteoliposomes (Figure 2A). We observed that Munc18−1 failed to activate the noncognate fusion reaction mediated by lysosomal/late endosomal SNAREs in the presence of Ficoll 70 (Figure 2B,C). We also cross-fused the v- and t-SNARE liposomes reconstituted with synaptic or lysosomal/late endosomal SNAREs in the presence of Ficoll 70 (Figure 2A). Again, none of these noncognate fusion reactions was stimulated by Munc18−1 (Figure 2B,C). These results indicate that the compartmental specificity of Munc18−1 is recapitulated in the crowded fusion assay and the specificity is dictated by its interactions with both the v- and t-SNAREs.

The Stimulatory Function of Munc18−1 Is Sensitive to Physiologically Relevant v-SNARE Mutations in Crowded Fusion Reactions. We next determined how the stimulatory function of Munc18−1 in the crowded fusion assay is affected by targeted mutations. Here, we focused on point mutations in the v-SNARE VAMP2 (Figure 3A). Munc18−1 can bind to multiple SNARE assemblies including the syntaxin monomer, the binary t-SNARE complex, and the ternary SNARE complex.[8b,c,22] By nature, only v-SNARE mutations can selectively interfere with the association of Munc18−1 with the ternary SNARE complex, whereas mutations in t-SNAREs or SM proteins may affect multiple SNARE-SM binding modes. We first examined two pairs of N-terminal mutations—L32A/T35A (M1) and V39A/V42A (M2)—that did not reduce exocytic vesicle fusion in vivo.[23] We observed that the stimulatory activity of Munc18−1 was not impacted by these VAMP2 mutations in crowded fusion reactions (Figure 3B,C), consistent with the effects of the mutations in the cell.[23] Similarly, a mutation in the zero layer of VAMP2—R56Q

DOI: 10.1021/jacs.5b08306
J. Am. Chem. Soc. 2015, 137, 12873−12883

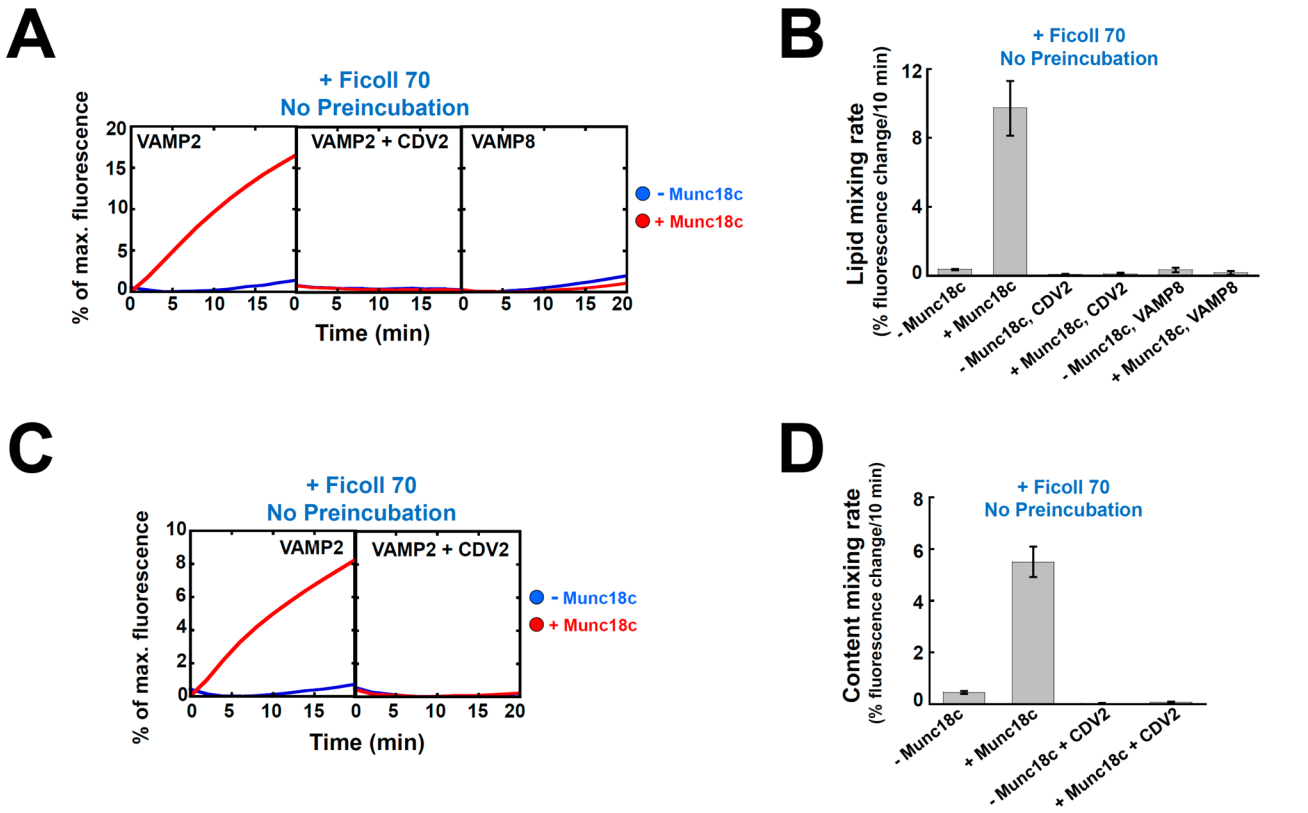


Figure 4. Munc18c strongly accelerates the kinetics of SNARE-dependent membrane fusion in the presence of crowding agents. (A) The t-SNARE liposomes containing syntaxin-4 and SNAP-23 were directed to fuse with VAMP2 liposomes in the absence or presence of 5 μM Munc18c. The reactions were carried out in the presence of 100 mg/mL Ficoll 70 without low-temperature preincubation. The fusion reactions were measured by the FRET-based lipid mixing assay. In negative controls, CDV2 was added to the reactions to the final concentration of 20 μM. (B) Initial lipid-mixing rates of the fusion reactions shown in A. Error bars indicate standard deviation. (C) The fusion reactions were performed as in A and measured by the content mixing assay. (D) Initial content-mixing rates of the fusion reactions shown in C. Error bars indicate standard deviation.

(M3)—did not interfere with the stimulatory function of Munc18−1 in crowded assays (Figure 3B,C), in agreement with the genetic observation that this point mutation resulted in normal synaptic releases in neurons.[24]

By contrast, mutations in any of the four tested C-terminal residues—L60 (M5), L63 (M6), L70 (M6) and F77 (M7)—abrogated the stimulatory activity of Munc18−1 in reconstituted fusion assays (Figure 3B,C). Interestingly, two of these C-terminal mutations—L70A and F77A—were known to strongly inhibit exocytosis in intact cells.[23] Thus, the stimulatory activity of Munc18−1 in the crowded fusion assay is sensitive to v-SNARE mutations and the results correlate with genetic observations (Table S1). Together, the results of compartmental specificity and v-SNARE mutations support our conclusion that the crowded fusion assay recapitulates the biological function of Munc18−1.

The Stimulatory Function of Munc18c in Reconstituted Fusion Reactions Is Also Dependent on Macromolecular Crowding. To establish the general role of macromolecular crowding in vesicle fusion, it is critical to examine a vesicle fusion pathway unrelated to synaptic exocytosis. To this end, we reconstituted a fusion reaction using SNAREs involved in insulin-regulated GLUT4 trafficking—syntaxin-4, SNAP-23, and VAMP2—and performed the fusion reactions in the presence of Ficoll 70. In uncrowded reconstituted assays, the stimulatory effect of the cognate SM protein Munc18c was also dependent on low-temperature preincubation with SNARE liposomes.[11a,18] In the presence of Ficoll 70, however, Munc18c potently stimulated both the lipid mixing and content mixing of the fusion reaction without requiring low-temperature preincubation (Figure 4A−D). Again, no content leakage was observed in the fusion reactions (Figure S8). Substitution of VAMP2 with VAMP8, a noncognate v-SNARE, abolished the stimulatory activity of Munc18c (Figure 4A,B). Thus, similar to the results of the Munc18−1 experiments, the stimulatory activity and compartmental specificity of Munc18c were recapitulated in the crowded fusion assay. These data suggest that the effect of macromolecular crowding on SNARE-SM mediated membrane fusion is conserved among intracellular vesicle fusion pathways.

The SNARE-SM Mediated Fusion Reaction Is Modulated by NSF and α-SNAP. Next, we sought to determine how the SNARE-SM mediated membrane fusion is influenced by NSF and α-SNAP. The well-established role of NSF and α-SNAP in vesicle fusion is to dissociate the postfusion *cis*-SNARE complex.[10] However, it is possible that NSF and α-SNAP may also influence the actions of SNAREs and SM proteins during membrane fusion. Accurate recapitulation of SM protein functions in the crowded fusion assay enabled us to examine the activities of NSF and α-SNAP in SNARE-SM mediated membrane fusion. NSF and α-SNAP were added to the Ficoll 70-containing fusion reaction (Figure 5A). We observed that the basal fusion was slightly enhanced by NSF and α-SNAP (Figure 5A,B). In a liposome coflotation assay, NSF and α-SNAP efficiently dissociated liposome-anchored *cis*-SNARE complexes (Figure S9), indicating that they were fully

DOI: 10.1021/jacs.5b08306
J. Am. Chem. Soc. 2015, 137, 12873−12883

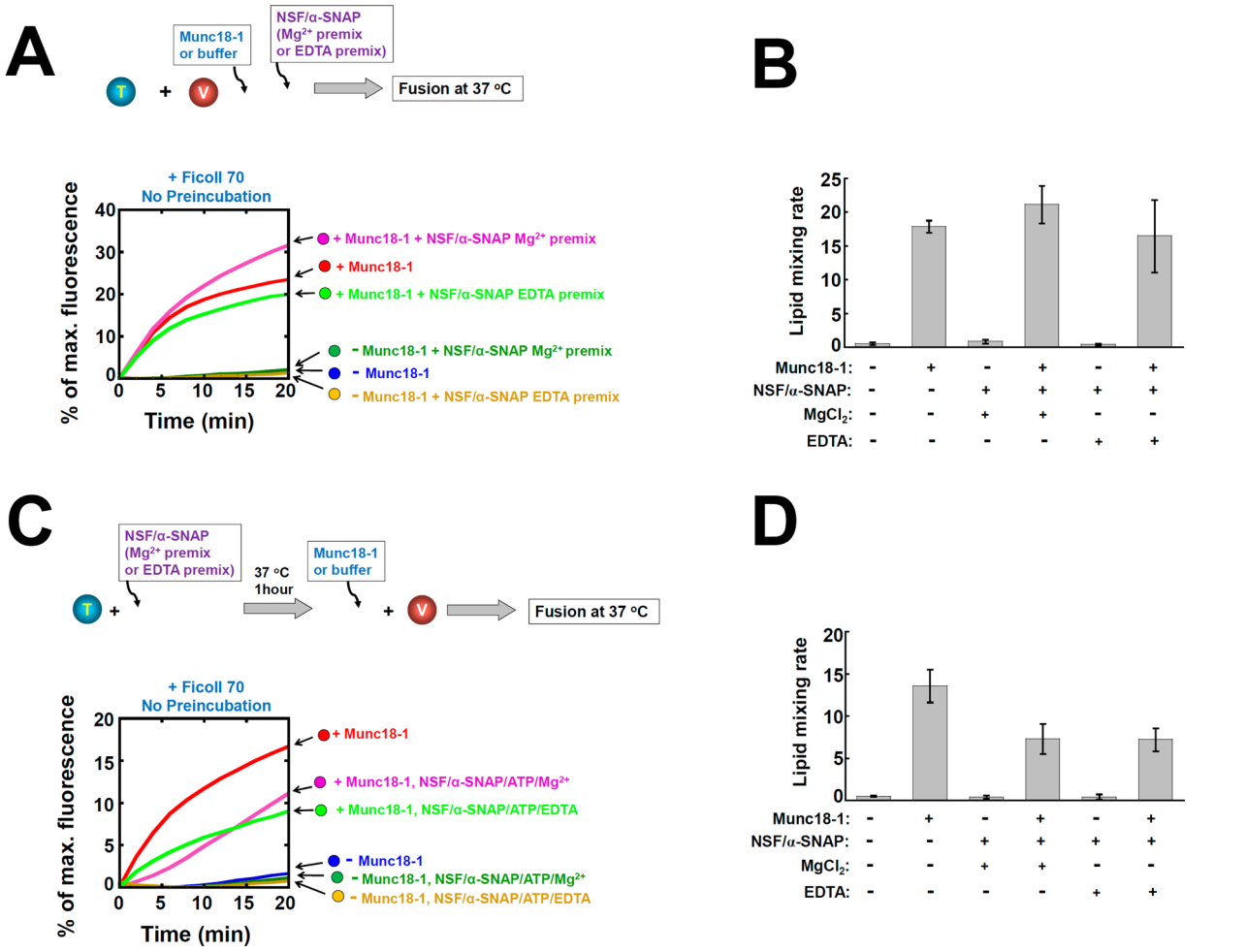


Figure 5. NSF and α-SNAP play dual role in SNARE-SM mediated membrane fusion. (A) Top: diagram illustrating the experimental procedure of the reconstituted fusion reactions. Bottom: reconstituted SNARE-dependent fusion reactions carried out in the presence of SNARE regulators. The t-SNARE liposomes containing syntaxin-1 and SNAP-25 were directed to fuse with VAMP2 liposomes in the absence or presence of 5 μM Munc18−1. To test the activities of NSF and α-SNAP, Mg^{2+}-premix or EDTA-premix was added to the fusion reactions. Ingredients of the samples were mixed and immediately loaded into a prewarmed microplate to initiate fusion. The fusion reactions were measured by the FRET-based lipid mixing assay. All reactions were carried out in the presence of 100 mg/mL Ficoll 70. (B) Initial lipid-mixing rates of the reconstituted fusion reactions shown in A. Error bars indicate standard deviation. (C) Top: diagram illustrating the experimental procedure of the reconstituted fusion reactions in which the t-SNARE liposomes were pretreated with NSF and α-SNAP. The fusion reactions were measured by the FRET-based lipid mixing assay. (D) Initial lipid-mixing rates of the reconstituted fusion reactions in C. Error bars indicate standard deviation.

active. Interestingly, the SNARE-Munc18−1 mediated fusion was also moderately increased in the presence of NSF and α-SNAP (Figure 5A,B). The increase in fusion rate was observed only in the presence of Mg^{2+} (Figure 5A), suggesting that it was dependent on the ATPase activity of NSF.

We then pretreated the t-SNARE liposomes with NSF and α-SNAP in order to examine their role in the early step of the fusion reaction. Munc18−1 and v-SNARE liposomes were subsequently added to initiate fusion (Figure 5C). We observed that the basal SNARE-mediated fusion was slightly reduced when the t-SNARE liposomes were pretreated with NSF and α-SNAP (Figure 5C,D). The inhibitory effects of NSF and α-SNAP were independent of the ATPase activity of NSF because removal of Mg^{2+} resulted in the same level of fusion decrease (Figure 5C,D). These results are consistent with the previous finding that α-SNAP itself can bind to the t-SNAREs and reduce the basal fusion.[25] We observed that the SNARE-Munc18−1 mediated fusion was also moderately reduced when the t-SNARE liposomes were pretreated with NSF and α-SNAP (Figure 5C,D). Again, the decrease in the fusion rate was independent of Mg^{2+} (Figure 5C,D). When normalized to the corresponding basal fusion rates, however, the stimulatory activities of Munc18−1 in these fusion reactions were comparable to those in the control reactions (Figure 5C,D). Together, these data demonstrate that NSF and α-SNAP both positively and negatively modulate the SNARE-SM mediated fusion reaction.

Mutations in the L60 or L63 Residue of the v-SNARE Inhibit Synaptic Exocytosis in Cultured Neurons. Finally, we sought to further examine the biological relevance of our findings. Seven VAMP2 mutations were tested in reconstituted fusion assays (Figure 3A). Five of these seven mutations were previously investigated in genetic studies and the data correlate well with our in vitro findings (Table S1). Our crowded assays showed that mutations in either the L60 or L63 residue of VAMP2 abrogated Munc18−1 activity but the effects of these mutations in vivo were still unclear.

DOI: 10.1021/jacs.5b08306
J. Am. Chem. Soc. 2015, 137, 12873−12883

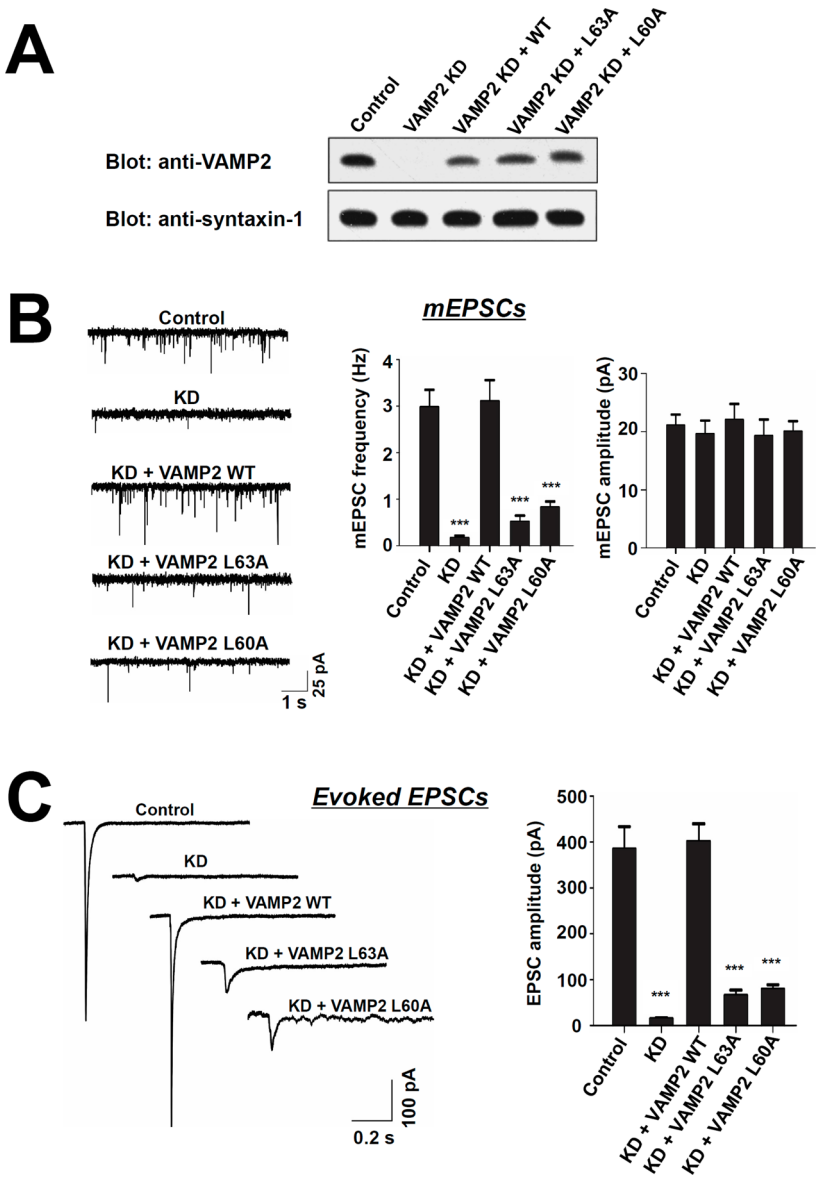


Figure 6. Synaptic exocytosis is inhibited by the L60A (M4) or L63A (M5) mutation in VAMP2. (A) Immunoblots showing the expression of VAMP2 and syntaxin-1 in the indicated neurons. VAMP2 was detected by monoclonal anti-VAMP2 antibodies (Cl69.1, Synaptic Systems) while syntaxin-1 was probed using monoclonal anti-syntaxin-1 antibodies (HPC-1, Synaptic Systems). Control: WT neurons without lentiviral infection. (B) Spontaneous synaptic vesicle fusion monitored as miniature excitatory postsynaptic currents (mEPSCs). Left: representative traces of mEPSCs. Middle: summary graph of mEPSC frequency. Right: summary graph of mEPSC amplitudes. (C) Left: representative traces of excitatory postsynaptic currents (EPSCs) evoked by isolated action potentials in cultured neurons. Right: summary graph of EPSC amplitudes. Data shown in the summary graphs are means ± SEMs. Numbers of neurons and independent cultures are listed in Table S2. Statistical analysis was performed using the Student's *t*-test comparing a test data set to the control experiment. *** $P < 0.001$.

To determine how the L60A (M4) and L63A (M5) mutations affect synaptic release, we introduced lentivirus-based shRNAs into cultured mouse neurons to silence VAMP2 expression. Immunoblotting analysis indicated that VAMP2 levels were reduced by >90% in the knockdown cells (Figure 6A). Using electrophysiological recordings, we observed that VAMP2 knockdown strongly diminished the frequency of spontaneous neurotransmitter release as monitored by the miniature excitatory postsynaptic currents (mEPSCs) (Figure 6B). WT VAMP2 fully rescued the phenotype, whereas the L60A or L63A mutant did not (Figure 6B). The amplitudes of mEPSCs were not changed by VAMP2 knockdown or layer mutations (Figure 6B).

We next measured evoked neurotransmitter release in the cultured neurons. VAMP2 knockdown resulted in a strong reduction in the EPSCs triggered by isolated action potentials (Figure 6C). The defects in evoked responses were fully rescued by WT VAMP2 but not by the L60A or L63A mutant (Figure 6C). Thus, mutations in either the L60 or L63 residue of VAMP2 strongly inhibit synaptic exocytosis, confirming the findings of our reconstitution experiments. These genetic data provide further physiological support for the role of macromolecular crowding in vesicle fusion.

■ DISCUSSION

In view of the profound effects of macromolecular crowding on macromolecular interactions,[26] it is surprising that crowding

DOI: 10.1021/jacs.5b08306
J. Am. Chem. Soc. 2015, 137, 12873−12883

agents have not been tested in reconstituted vesicle fusion assays. In this work, we established a crucial role of macromolecular crowding in the SNARE-SM mediated fusion reaction. While the basal SNARE-mediated fusion is only slightly enhanced by macromolecular crowding, likely due to the intrinsically high binding affinity between the v- and t-SNAREs, the stimulatory activities of SM proteins in reconstituted assays are fully dependent on the presence of crowding agents. The biological relevance of our crowded fusion assays is supported by multiple lines of evidence: (1) the potency of SM protein activities; (2) bypassing of the artificial low-temperature preincubation step; (3) compartmental specificity of SM protein activities; and (4) correlation with genetic observations.

While supplying the energy for membrane merging, SNAREs alone are incapable of mediating efficient vesicle fusion and a cognate SM protein is needed to accelerate the fusion kinetics. SM proteins stimulate membrane fusion by recognizing the cognate *trans*-SNARE complex and accelerating its zippering. Assembled between apposed lipid bilayers, the *trans*-SNARE complex is a highly dynamic structure that cannot properly associate with the SM protein in an uncrowded environment. With macromolecular crowding, however, the *trans*-SNARE-SM association is dramatically enhanced due to the macromolecular crowding effect, allowing the SM protein to act in concert with SNAREs to drive membrane fusion.

Comparative analysis performed in this study suggests that in general our previous conclusions derived from uncrowded assays (with low-temperature preincubation) remain valid. We posit that the artificial low-temperature preincubation accumulates and stabilizes *trans*-SNARE intermediates, thus achieving a similar result as the addition of macromolecular crowding agents. In addition to the conserved function investigated in this study, a SM protein often exhibits pathway-specific activities. For instance, Munc18−1 binds to the "closed" syntaxin-1 monomer, a binding mode not conserved in other SM proteins.[22,27] This closed syntaxin binding mode positively regulates syntaxin trafficking as well as the early steps of SNARE assembly.[15b,28] Since the Munc18−1-syntaxin-1 binding mode is of high affinity and readily observed in uncrowded assays,[22] it is expected to be less influenced by macromolecular crowding.

In addition to their well-established postfusion role in *cis*-SNARE disassembly, NSF and α-SNAP also modulate the SNARE-SM mediated fusion reaction. First, α-SNAP can bind to the t-SNAREs and reduces fusion rate, an activity independent of NSF. After the *trans*-SNARE complex begins to assemble, NSF and α-SNAP increase the fusion rate, a function requiring both α-SNAP and the ATPase activity of NSF. The increases in fusion kinetics may be due to the recycling *cis*-SNARE complexes formed during the reaction such that more free v- and t-SNAREs are available for driving fusion. Alternatively, NSF and α-SNAP may dissociate unproductive SNARE assemblies such as antiparallel SNARE complexes.[29] It should be noted, while NSF and α-SNAP influence the membrane fusion kinetics, their effects on the prefusion steps are modest and the fusion rate is still largely determined by the activities of SNAREs and SM proteins. In the presence of pathway-specific factors, NSF and/or α-SNAP also play additional roles in fusion regulation.[28a,30] The overall effects of NSF and α-SNAP are likely dictated by the dynamic balance of these activities, dependent on their local concentrations as well as the duration they can access a SNARE assembly.

In this study, we focused on the conserved molecules involved in all vesicle fusion pathways. The next challenge in the field is to include specialized regulatory factors that are not conserved among vesicle fusion pathways. For example, the fusion of synaptic vesicles with the plasma membrane (synaptic exocytosis) requires a number of exocytosis-specific factors such as synaptotagmin and complexin.[4b,31] These specialized regulators appeared later in evolution and their functions were evolved to be compatible with the conserved fusion machinery. Hence, whereas SNAREs and SM proteins can be studied in the absence of pathway-specific factors, a reconstituted system designed to investigate specialized factors needs to incorporate both SNAREs and SM proteins. Our preliminary studies indicate that Munc18−1 also strongly stimulated membrane fusion in the presence of synaptotagmin-1 and complexin-1 (Figure S10). Mutations in the v-SNARE abolished the stimulatory function of Munc18−1 (Figure S10),[8b] whereas the activities of synaptotagmin-1 and complexin-1 were unaffected (Figure S10). Membrane-anchored SNAREs and regulators can contribute to the overall macromolecular crowding at the fusion sites. However, the stimulatory activities of Munc18−1 were still fully dependent on macromolecular crowding agents in reconstituted assays, suggesting that vesicle fusion proteins themselves are insufficient to induce the macromolecular crowding effect required for SM protein function. In future reconstitution studies, substantial efforts are needed to fully recapitulate the temporal and spatial organization of the regulatory factors along the dynamic SNARE assembly pathway.

On the basis of the findings of this and other studies, we suggest that the following criteria can be taken into consideration in future reconstitution studies of vesicle fusion. First of all, the content mixing of a fusion reaction needs to be examined because under certain conditions lipid mixing signals might result from liposome tethering rather than true membrane fusion. Moreover, irrespective of the detection method (bulk or single liposome), content leakage controls should be included to examine the integrity of liposome membranes during fusion reactions. Second, whenever applicable, the compartmental specificity of a regulatory factor needs to be tested using noncognate SNARE isoforms. This simple but powerful experiment is often more advantageous over point mutations for cross-examining the physiological relevance of a finding. Point mutations often only partially reduce protein−protein binding affinity and may compromise the folding of a protein. Finally, a reconstituted fusion system needs to include macromolecular crowding agents to mimic the crowded intracellular environment. In this regard, macromolecular crowding is of the same importance as other controlled parameters of a reconstituted assay such as pH and ionic strength.

■ CONCLUSION

Two key conclusions can be drawn from the findings of our crowded fusion assays. First, the vesicle fusion machinery has been evolved to function optimally within the crowded environment of the cell. SNAREs and SM proteins actually take advantage of intracellular macromolecular crowding to efficiently drive vesicle fusion. Second, the SNARE-SM mediated vesicle fusion can be reconstituted and characterized only in the presence of macromolecular crowding agents.

DOI: 10.1021/jacs.5b08306
J. Am. Chem. Soc. 2015, 137, 12873−12883

Membrane and secretory proteins constitute about one-third of total proteins in eukaryotic cells. These proteins rely on vesicular transport to reach their destined cellular compartments. Hence, macromolecular crowding is not only important to the vesicle fusion process but also plays a central role in the maintenance and propagation of the entire internal membrane network of eukaryotic cells.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b08306.

Additional figures (Figures S1−10). Supporting tables (Tables S1 and S2). (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*jingshi.shen@colorado.edu

Present Address

†Applied and Engineering Physics Cornell University Ithaca, New York 14853, United States.

Author Contributions

‡H.Y. and S.S.R. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the members of the Shen laboratory for helpful discussions. This work was supported by National Institutes of Health grants (GM102217 and DK095367) to JS, a Pew Scholar Award to JS, and a Cancer League of Colorado seed grant to JS.

■ REFERENCES

(1) (a) Ellis, R. J. *Curr. Opin. Struct. Biol.* **2001**, *11* (1), 114−9. (b) Zhou, H. X.; Rivas, G.; Minton, A. P. *Annu. Rev. Biophys.* **2008**, *37*, 375−97.

(2) (a) Luh, L. M.; Hänsel, R.; Löhr, F.; Kirchner, D. K.; Krauskopf, K.; Pitzius, S.; Schäfer, B.; Tufar, P.; Corbeski, I.; Güntert, P.; Dötsch, V. *J. Am. Chem. Soc.* **2013**, *135* (37), 13796−13803. (b) Kurniawan, N. A.; Enemark, S.; Rajagopalan, R. *J. Am. Chem. Soc.* **2012**, *134* (24), 10200−10208. (c) Kilburn, D.; Roh, J. H.; Behrouzi, R.; Briber, R. M.; Woodson, S. A. *J. Am. Chem. Soc.* **2013**, *135* (27), 10055−10063. (d) Hong, J.; Gierasch, L. M. *J. Am. Chem. Soc.* **2010**, *132* (30), 10445−10452. (e) Harada, R.; Tochio, N.; Kigawa, T.; Sugita, Y.; Feig, M. *J. Am. Chem. Soc.* **2013**, *135* (9), 3696−3701. (f) Denesyuk, N. A.; Thirumalai, D. *J. Am. Chem. Soc.* **2011**, *133* (31), 11858−11861.

(3) (a) Homouz, D.; Perham, M.; Samiotakis, A.; Cheung, M. S.; Wittung-Stafshede, P. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105* (33), 11754−9. (b) Kuznetsova, I. M.; Turoverov, K. K.; Uversky, V. N. *Int. J. Mol. Sci.* **2014**, *15* (12), 23090−140.

(4) (a) Wickner, W.; Schekman, R. *Nat. Struct. Mol. Biol.* **2008**, *15* (7), 658−64. (b) Rizo, J.; Sudhof, T. C. *Annu. Rev. Cell Dev. Biol.* **2012**, *28*, 279−308. (c) Sollner, T.; Whiteheart, S. W.; Brunner, M.; Erdjument-Bromage, H.; Geromanos, S.; Tempst, P.; Rothman, J. E. *Nature* **1993**, *362* (6418), 318−24. (d) Kozlov, M. M.; McMahon, H. T.; Chernomordik, L. V. *Trends Biochem. Sci.* **2010**, *35* (12), 699−706. (e) Ohya, T.; Miaczynska, M.; Coskun, U.; Lommer, B.; Runge, A.; Drechsel, D.; Kalaidzidis, Y.; Zerial, M. *Nature* **2009**, *459* (7250), 1091−7. (f) Rizo, J.; Xu, J. *Annu. Rev. Biophys.* **2015**, *44*, 339−67.

(5) (a) Sutton, R. B.; Fasshauer, D.; Jahn, R.; Brunger, A. T. *Nature* **1998**, *395* (6700), 347−53. (b) Stein, A.; Weber, G.; Wahl, M. C.; Jahn, R. *Nature* **2009**, *460* (7254), 525−8.

(6) (a) Weber, T.; Zemelman, B. V.; McNew, J. A.; Westermann, B.; Gmachl, M.; Parlati, F.; Sollner, T. H.; Rothman, J. E. *Cell* **1998**, *92* (6), 759−72. (b) Jahn, R.; Scheller, R. H. *Nat. Rev. Mol. Cell Biol.* **2006**, *7* (9), 631−43. (c) Katz, L.; Brennwald, P. *Molecular biology of the cell* **2000**, *11* (11), 3849−58.

(7) (a) Melia, T. J.; Weber, T.; McNew, J. A.; Fisher, L. E.; Johnston, R. J.; Parlati, F.; Mahal, L. K.; Sollner, T. H.; Rothman, J. E. *J. Cell Biol.* **2002**, *158* (5), 929−40. (b) Pobbati, A. V.; Stein, A.; Fasshauer, D. *Science (Washington, DC, U. S.)* **2006**, *313* (5787), 673−6. (c) Gao, Y.; Zorman, S.; Gundersen, G.; Xi, Z.; Ma, L.; Sirinakis, G.; Rothman, J. E.; Zhang, Y. *Science (Washington, DC, U. S.)* **2012**, *337* (6100), 1340−3. (d) Li, F.; Kummel, D.; Coleman, J.; Reinisch, K. M.; Rothman, J. E.; Pincet, F. *J. Am. Chem. Soc.* **2014**, *136* (9), 3456−64.

(8) (a) Hata, Y.; Slaughter, C. A.; Sudhof, T. C. *Nature* **1993**, *366* (6453), 347−51. (b) Shen, J.; Tareste, D. C.; Paumet, F.; Rothman, J. E.; Melia, T. J. *Cell* **2007**, *128* (1), 183−95. (c) Dulubova, I.; Khvotchev, M.; Liu, S.; Huryeva, I.; Sudhof, T. C.; Rizo, J. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104* (8), 2697−702.

(9) (a) Zhao, M.; Wu, S.; Zhou, Q.; Vivona, S.; Cipriano, D. J.; Cheng, Y.; Brunger, A. T. *Nature* **2015**, *518* (7537), 61−7. (b) Chang, L. F.; Chen, S.; Liu, C. C.; Pan, X.; Jiang, J.; Bai, X. C.; Xie, X.; Wang, H. W.; Sui, S. F. *Nat. Struct. Mol. Biol.* **2012**, *19* (3), 268−75. (c) Rothman, J. E. *Nature* **1994**, *372* (6501), 55−63.

(10) Sudhof, T. C.; Rothman, J. E. *Science (Washington, DC, U. S.)* **2009**, *323* (5913), 474−7.

(11) (a) Yu, H.; Rathore, S. S.; Lopez, J. A.; Davis, E. M.; James, D. E.; Martin, J. L.; Shen, J. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110* (35), E3271−80. (b) Toonen, R. F.; Verhage, M. *Trends Neurosci.* **2007**, *30* (11), 564−72.

(12) (a) Tellam, J. T.; McIntosh, S.; James, D. E. *J. Biol. Chem.* **1995**, *270* (11), 5857−63. (b) Verhage, M.; Maia, A. S.; Plomp, J. J.; Brussaard, A. B.; Heeroma, J. H.; Vermeer, H.; Toonen, R. F.; Hammer, R. E.; van den Berg, T. K.; Missler, M.; Geuze, H. J.; Sudhof, T. C. *Science (Washington, DC, U. S.)* **2000**, *287* (5454), 864−9. (c) Weimer, R. M.; Richmond, J. E.; Davis, W. S.; Hadwiger, G.; Nonet, M. L.; Jorgensen, E. M. *Nat. Neurosci.* **2003**, *6* (10), 1023−30.

(13) Shen, J.; Rathore, S.; Khandan, L.; Rothman, J. E. *J. Cell Biol.* **2010**, *190* (1), 55−63.

(14) (a) Carr, C. M.; Grote, E.; Munson, M.; Hughson, F. M.; Novick, P. J. *J. Cell Biol.* **1999**, *146* (2), 333−44. (b) Scott, B. L.; Van Komen, J. S.; Irshad, H.; Liu, S.; Wilson, K. A.; McNew, J. A. *J. Cell Biol.* **2004**, *167* (1), 75−85. (c) Munson, M.; Chen, X.; Cocina, A. E.; Schultz, S. M.; Hughson, F. M. *Nat. Struct. Biol.* **2000**, *7* (10), 894−902. (d) Kramer, L.; Ungermann, C. *Molecular biology of the cell* **2011**, *22* (14), 2601−11. (e) Pieren, M.; Schmidt, A.; Mayer, A. *Nat. Struct. Mol. Biol.* **2010**, *17* (6), 710−7. (f) Carpp, L. N.; Ciufo, L. F.; Shanks, S. G.; Boyd, A.; Bryant, N. J. *J. Cell Biol.* **2006**, *173* (6), 927−36.

(15) (a) Rathore, S. S.; Bend, E. G.; Yu, H.; Hammarlund, M.; Jorgensen, E. M.; Shen, J. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107* (52), 22399−406. (b) Shi, L.; Kummel, D.; Coleman, J.; Melia, T. J.; Giraudo, C. G. *Molecular biology of the cell* **2011**, *22* (21), 4150−60. (c) Tareste, D.; Shen, J.; Melia, T. J.; Rothman, J. E. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105* (7), 2380−5. (d) Rathore, S. S.; Ghosh, N.; Ouyang, Y.; Shen, J. *Molecular biology of the cell* **2011**, *22* (14), 2612−9. (e) Rodkey, T. L.; Liu, S.; Barry, M.; McNew, J. A. *Molecular biology of the cell* **2008**, *19* (12), 5422−34. (f) Schollmeier, Y.; Krause, J. M.; Kreye, S.; Malsam, J.; Sollner, T. H. *J. Biol. Chem.* **2011**, *286* (35), 30582−90. (g) Diao, J.; Su, Z.; Lu, X.; Yoon, T. Y.; Shin, Y. K.; Ha, T. *ACS Chem. Neurosci.* **2010**, *1* (3), 168−174.

(16) Weber, T.; Parlati, F.; McNew, J. A.; Johnston, R. J.; Westermann, B.; Sollner, T. H.; Rothman, J. E. *J. Cell Biol.* **2000**, *149* (5), 1063−72.

(17) Latham, C. F.; Lopez, J. A.; Hu, S. H.; Gee, C. L.; Westbury, E.; Blair, D. H.; Armishaw, C. J.; Alewood, P. F.; Bryant, N. J.; James, D. E.; Martin, J. L. *Traffic* **2006**, *7* (10), 1408−19.

(18) (a) Yu, H.; Rathore, S. S.; Davis, E. M.; Ouyang, Y.; Shen, J. *Molecular biology of the cell* **2013**, *24* (8), 1176−84. (b) Yu, H.; Rathore, S. S.; Shen, J. *J. Biol. Chem.* **2013**, *288* (26), 18885−93.

DOI: 10.1021/jacs.5b08306
J. Am. Chem. Soc. 2015, 137, 12873−12883

(19) Yu, H.; Rathore, S. S.; Gulbranson, D. R.; Shen, J. *J. Biol. Chem.* **2014**, *289* (37), 25571−80.
(20) Davis, E. M.; Kim, J.; Menasche, B. L.; Sheppard, J.; Liu, X.; Tan, A. C.; Shen, J. *Cell Rep.* **2015**, *11* (11), 1727−36.
(21) (a) Zhang, Y.; Pak, C.; Han, Y.; Ahlenius, H.; Zhang, Z.; Chanda, S.; Marro, S.; Patzke, C.; Acuna, C.; Covy, J.; Xu, W.; Yang, N.; Danko, T.; Chen, L.; Wernig, M.; Sudhof, T. C. *Neuron* **2013**, *78* (5), 785−98. (b) Maximov, A.; Tang, J.; Yang, X.; Pang, Z. P.; Sudhof, T. C. *Science (Washington, DC, U. S.)* **2009**, *323* (5913), 516−21.
(22) (a) Misura, K. M.; Scheller, R. H.; Weis, W. I. *Nature* **2000**, *404* (6776), 355−62. (b) Burkhardt, P.; Hattendorf, D. A.; Weis, W. I.; Fasshauer, D. *EMBO J.* **2008**, *27* (7), 923−33.
(23) Walter, A. M.; Wiederhold, K.; Bruns, D.; Fasshauer, D.; Sorensen, J. B. *J. Cell Biol.* **2010**, *188* (3), 401−13.
(24) Deak, F.; Shin, O. H.; Kavalali, E. T.; Sudhof, T. C. *J. Neurosci.* **2006**, *26* (25), 6668−76.
(25) Park, Y.; Vennekate, W.; Yavuz, H.; Preobraschenski, J.; Hernandez, J. M.; Riedel, D.; Walla, P. J.; Jahn, R. *J. Biol. Chem.* **2014**, *289* (23), 16326−35.
(26) (a) White, D. A.; Buell, A. K.; Knowles, T. P. J.; Welland, M. E.; Dobson, C. M. *J. Am. Chem. Soc.* **2010**, *132* (14), 5170−5175. (b) Wang, Y.; Sarkar, M.; Smith, A. E.; Krois, A. S.; Pielak, G. J. *J. Am. Chem. Soc.* **2012**, *134* (40), 16614−16618. (c) Stagg, L.; Christiansen, A.; Wittung-Stafshede, P. *J. Am. Chem. Soc.* **2011**, *133* (4), 646−648. (d) Sotomayor-Pérez, A.-C.; Subrini, O.; Hessel, A.; Ladant, D.; Chenal, A. *J. Am. Chem. Soc.* **2013**, *135* (32), 11929−11934. (e) Senske, M.; Törk, L.; Born, B.; Havenith, M.; Herrmann, C.; Ebbinghaus, S. *J. Am. Chem. Soc.* **2014**, *136* (25), 9036−9041.
(27) Latham, C. F.; Osborne, S. L.; Cryle, M. J.; Meunier, F. A. *J. Neurochem.* **2007**, *100* (6), 1543−54.
(28) (a) Ma, C.; Su, L.; Seven, A. B.; Xu, Y.; Rizo, J. *Science (Washington, DC, U. S.)* **2013**, *339* (6118), 421−5. (b) Arunachalam, L.; Han, L.; Tassew, N. G.; He, Y.; Wang, L.; Xie, L.; Fujita, Y.; Kwan, E.; Davletov, B.; Monnier, P. P.; Gaisano, H. Y.; Sugita, S. *Mol. Biol. Cell* **2008**, *19* (2), 722−34.
(29) (a) Weninger, K.; Bowen, M. E.; Chu, S.; Brunger, A. T. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100* (25), 14800−5. (b) Lobingier, B. T.; Nickerson, D. P.; Lo, S. Y.; Merz, A. J. *eLife* **2014**, *3*, e02272.
(30) Zick, M.; Orr, A.; Schwartz, M. L.; Merz, A. J.; Wickner, W. T. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112* (18), E2290−7.
(31) (a) James, D. J.; Kowalchyk, J.; Daily, N.; Petrie, M.; Martin, T. F. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (41), 17308−13. (b) Chapman, E. R. *Annu. Rev. Biochem.* **2008**, *77*, 615−41. (c) Shin, O. H.; Xu, J.; Rizo, J.; Sudhof, T. C. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (38), 16469−74. (d) Zhou, K. M.; Dong, Y. M.; Ge, Q.; Zhu, D.; Zhou, W.; Lin, X. G.; Liang, T.; Wu, Z. X.; Xu, T. *Neuron* **2007**, *56* (4), 657−69. (e) Kummel, D.; Krishnakumar, S. S.; Radoff, D. T.; Li, F.; Giraudo, C. G.; Pincet, F.; Rothman, J. E.; Reinisch, K. M. *Nat. Struct. Mol. Biol.* **2011**, *18* (8), 927−33.

DOI: 10.1021/jacs.5b08306
J. Am. Chem. Soc. 2015, 137, 12873−12883