

RESEARCH ARTICLE

Differential Effects of Munc18s on Multiple Degranulation-Relevant Trans-SNARE Complexes

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OPEN ACCESS

Citation: Xu H, Arnold MG, Kumar SV (2015) Differential Effects of Munc18s on Multiple Degranulation-Relevant Trans-SNARE Complexes. PLoS ONE 10(9): e0138683. doi:10.1371/journal.pone.0138683

Editor: Hong Wanjin, Institute of Molecular and Cell Biology, Biopolis, UNITED STATES

Received: June 3, 2015

Accepted: September 2, 2015

Published: September 18, 2015

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: HX received The University of Southern Mississippi (<http://www.usm.edu>) Development Grant DE01475 for the study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Mast cell exocytosis, which includes compound degranulation and vesicle-associated piecemeal degranulation, requires multiple Q- and R- SNAREs. It is not clear how these SNAREs pair to form functional trans-SNARE complexes and how these trans-SNARE complexes are selectively regulated for fusion. Here we undertake a comprehensive examination of the capacity of two Q-SNARE subcomplexes (syntaxin3/SNAP-23 and syntaxin4/SNAP-23) to form fusogenic trans-SNARE complexes with each of the four granule-borne R-SNAREs (VAMP2, 3, 7, 8). We report the identification of at least six distinct trans-SNARE complexes under enhanced tethering conditions: i) VAMP2/syntaxin3/SNAP-23, ii) VAMP2/syntaxin4/SNAP-23, iii) VAMP3/syntaxin3/SNAP-23, iv) VAMP3/syntaxin4/SNAP-23, v) VAMP8/syntaxin3/SNAP-23, and vi) VAMP8/syntaxin4/SNAP-23. We show for the first time that Munc18a operates synergistically with SNAP-23-based non-neuronal SNARE complexes (i to iv) in lipid mixing, in contrast to Munc18b and c, which exhibit no positive effect on any SNARE combination tested. Pre-incubation with Munc18a renders the SNARE-dependent fusion reactions insensitive to the otherwise inhibitory R-SNARE cytoplasmic domains, suggesting a protective role of Munc18a for its cognate SNAREs. Our findings substantiate the recently discovered but unexpected requirement for Munc18a in mast cell exocytosis, and implicate post-translational modifications in Munc18b/c activation.

Introduction

Mast cells play critical roles in immunity and allergic inflammation through regulated release of various biologically active mediators (e.g., histamine, proteases, and cytokines) [1]. Many of these mediators are pre-stored in secretory lysosomes/granules that, upon mast cell activation, undergo signaling-dependent compound degranulation (homotypic fusion with one another and heterotypic fusion to the plasma membrane) [2] or piecemeal degranulation (granule-associated cargos are packaged into small vesicles that subsequently transport to and fuse with the

cell surface) [3]. Like all fusion events along the endocytic and secretory pathways, mast cell exocytosis requires SNAREs {SNAP [soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein] receptors} anchored to apposed membranes to form a fusogenic 4-helical bundle, the so-called trans-SNARE complex [4]. A functional or fusogenic trans-SNARE complex is typically formed by three Q-SNAREs (Qa, b, c) emanating from one membrane and one R-SNARE from the other [4]. However, for mast cell exocytosis, the nature of the underlying trans-SNARE complex(es) has been elusive, in part due to the presence of quite a number of degranulation-relevant SNAREs [5,6,7,8,9,10,11,12,13].

Localization studies of resting murine mast cells indicate that secretory lysosomes/granules are decorated with R-SNAREs VAMP2, 3, 7, 8 [6,7,12,13,14,15] and Qa-SNARE syntaxin3 [7,12,13,14], whereas the plasma membrane is enriched with Qa-SNARE syntaxin4 [13,16] and Qb,c-SNARE SNAP-23 [6,13]. During mast cell compound degranulation, syntaxin3 relocates from the secretory lysosomes to the plasma membrane [9], whereas SNAP-23 relocates from the plasma membrane to the secretory lysosomes [13]. According to the 3Q: 1R rule [17], these seven SNAREs can potentially form eight distinct trans-SNARE complexes. Among them, VAMP8 (R)/syntaxin4 (Qa)/SNAP-23 (Qbc) represents best established trans-SNARE complex for mast cell degranulation [18], supported by degranulation assays involving knockout mice, primary cells and cultured cell lines [5,6,7,10,11,13,15,19], biochemical characterizations [7,10,19,20] and reconstitution studies [21,22]. However, multiple trans-SNARE complexes are required in activated mast cells to fulfill a number of roles. First, the homotypic and heterotypic fusion in degranulation may each exploit a distinct trans-SNARE complex. A particularly promising candidate for the homotypic granule fusion is syntaxin3, which appears to be the only known Qa-SNARE on the secretory lysosomes. It effectively binds SNAP-23 and VAMP8 [20] and is critical for optimal secretion of β -hexosaminidase in RBL (rat basophilic leukemia)-2H3 cells, a tumor analog of mucosal mast cells [9]. Second, piecemeal degranulation may play a prominent role under conditions where compound exocytosis is compromised and might exploit R-SNAREs other than VAMP8, as suggested by the lack of complete inhibition of β -hexosaminidase release in VAMP8-knockout mast cells [7,15]. In accordance with this, increasing amounts of VAMP2 and VAMP3 were found in association with SNAP-23 in VAMP8 deficient cells [7,8], suggesting a VAMP2- or VAMP3- based trans-SNARE complex might account for the remaining secretory activity. Third, there is heterogeneity within the secretory lysosome population in mast cells [23]. A recent study using mast cells derived from VAMP8-knockout mice showed that while VAMP8 is required for the regulated release of β -hexosaminidase and serotonin, it is dispensable for TNF- α and histamine secretion [15]. Thus, VAMP8-independent trans-SNARE complex(es) must exist for regulated degranulation in these cells. A leading alternative to VAMP8, besides VAMP2 and VAMP3, is VAMP7, which has been shown to mediate granule exocytosis in mature human mast cells [10].

The involvement of multiple exocytic trans-SNARE complexes in mast cell is in line with the observation that all three mammalian Munc18 isoforms specific for regulated exocytosis are linked to mast cell degranulation. Munc18s are members of the conserved Sec1-Munc18 (SM) protein family that regulate fusion by exploiting different modes of association with the fusion machinery [24,25]. Noted for its ability to activate the neuronal trans-SNARE complex [26], Munc18a has been thought to function mainly in synaptic transmission, but it is also expressed in non-neuronal tissues [27,28,29]. Very recently, a double knockdown of Munc18a and Munc18b in RBL cells was found to eliminate β -hexosaminidase release, whereas reintroducing Munc18a alone fully rescued the secretion defect [30]. However, through which set of SNAREs Munc18a operates in mast cell exocytosis is not clear. The functional requirement for Munc18b in mast cell exocytosis has long been recognized [16,31], but it was recently that its participation in microtubule-dependent granule translocation, a stage preceding granule

fusion, was delineated [9]. Whether Munc18b acts exclusively in translocation during mast cell degranulation or multitasks in both translocation and fusion awaits further clarification. Munc18c is ubiquitously expressed in mammals and interacts with syntaxin4 in a wide range of cells including RBL cells [31]. Although both negative and positive roles of Munc18c in SNARE-mediated GLUT4 exocytosis have been observed [32,33,34,35] [36], the functional importance of Munc18c in mast cell exocytosis remains to be established.

In this study, we used reconstitution to explore the functional pairing of each of the four granule-borne R-SNAREs (VAMP2, 3, 7, 8) with Q-SNAREs syntaxin3, syntaxin4, and SNAP-23. We then investigated the effects of three Munc18 isoforms respectively in each of the reconstituted fusion reactions. We report that Munc18a selectively promotes the lipid mixing mediated by VAMP2 and VAMP3, in a fashion that prevents the inhibitory action of R-SNARE cytoplasmic domains.

Materials and Methods

cDNA Constructs

The cDNA encoding rat SNAP-23 in the pGEX vector (gift from Paul Roche) was amplified using PCR and inserted in between the NcoI and EcoRI sites of the pMBP-parallel1 vector [37] to generate pMBP-TCS (TEV Cleavable Site)-SNAP23. Similarly, pMBP-TCS-Syx3 (rat), pMBP-TCS-Syx4 (rat), pMBP-TCS-VAMP2 (rat), pMBP-TCS-VAMP2CD (rat) pMBP-TCS-VAMP3 (rat), pMBP-TCS-VAMP8 (rat) and pMBP-TCS-VAMP8CD (rat) were generated respectively from pGEX-syntaxin3 (gift from Reinhard Jahn), UB339 (Syx4 construct; gift from Ulrich Blank), pGEX-Syb1-116 (gift from Jose Rizo), rat reference cDNA (for VAMP3; Zyagen), and pGEX-KG-endobrevin (gift from Reinhard Jahn). These constructs contain a 4-aa-long linker sequence (GAMG) between the TCS and the start codon of each SNARE for efficient cleavage by TEV, except the VAMP8 constructs which contain a 2-aa-long linker (GA). The cDNA for VAMP7 was amplified from rat reference cDNA and inserted into the LIC site of pET MBP His₆ LIC cloning vector (gift from Scott Gradia; Addgene plasmid # 37237) to generate pET-VAMP7-TCS-MBP-His₆. The cDNA for rat Munc18a was purchased from ThermoScientific (Clone ID #7315868), amplified using PCR and inserted in between the EcoRI and Sall sites of pMBP-parallel1 to generate pMBP-TCS-Munc18a. Rat Munc18b cDNA was generated from pCMV-Munc18-2 (gift from Thomas Südhof) and the rat reference cDNA using overlapping PCR to mitigate a point mutation near the 3' end in pCMV-Munc18-2 and an insertion mutation near the 5' end in the reference cDNA. The PCR product containing the correct sequence of Munc18b was initially insert into the EcoRI/Sall sites of pMBP-parallel1 for bacterial expression but later subcloned into the BamHI/Sall sites of pFAST-BAC-HT-JS (gift from Jingshi Shen) for insect cell line expression. All the cDNA constructs above were verified via DNA sequencing. Sequences of cloning primers are in [S1 Table](#). pET28a-NSF (hamster) and pET28a- α SNAP (cow) were kind gifts from Reinhard Jahn. pFL-38His₆-TEV was a kind gift from William Wickner. pFAST-BAC-HT-JS-Munc18c (mouse), pET28a-syntaxin4 and pET15b-SNAP23 were kind gifts from Dr. Jingshi Shen).

Proteins

All recombinant proteins purified in this study were quantified using the Bradford assay (BioRad) according to manufacturer's instruction, snap-frozen in small aliquots in liquid N₂, and stored at -70°C. To purify MBP-tagged SNAREs, *E. coli* Rosetta2 (Novagen) transformed with the respective plasmid was inoculated into 100 mL LB medium containing 100 μ g/mL ampicillin and 25 μ g/mL chloramphenicol. Following overnight growth at 37°C, the culture was added to 1L of Terrific Broth (TB) medium containing 100 μ g/mL ampicillin and 25 μ g/mL

chloramphenicol, and shaken at 37°C until the $OD_{600} \approx 1.5$. IPTG (1M) was added to a final concentration of 0.5mM. After 4 hours of continual growth at 37°C, bacteria were harvested by centrifugation (5k rpm, 5 min, room temperature, GS3 rotor). The pellet was resuspended in 20 mL of Buffer A (50mM HEPES-KOH, pH 7.5, 0.5M KCl, 10% glycerol, 1mM DTT) containing 5mM benzamidine, 1mM PMSF, 1x PIC (0.62 μ g/mL leupeptin, 4 μ g/mL pepstatin A, and 24.4 μ g/mL pefabloc-SC), and subject to two passes through a French Press at 900 psi. Except for SNAP-23, VAMP2cd and VAMP8cd, one-tenth volume of 1M n-octyl- β -D-glucoside (β -OG; Affymetrix) was added and incubation was continued at 4°C for 1 h with nutation. The lysates were then centrifuged at 40 k rpm for 1 h in a Beckman 70Ti rotor. The supernatants were added to 4mL of amylose resin (NEB) pre-equilibrated with the wash buffer (50mM HEPES-KOH, pH 7.5, 0.3M KCl, 10% glycerol and 0.1M β -OG). Following 1 h nutation at 4°C, the resin was packed into an empty Biorad Econo-column at 4°C, washed with 40mL of wash buffer, and eluted with 10mM maltose in wash buffer. MBP-SNAP-23, MBP-VAMP2cd and MBP-VAMP8cd were purified in the same fashion except without introducing β -OG in the wash and elution buffer. To purify untagged syntaxin4 and His₆-SNAP-23, BL21(DE3) co-transformed with pET28a-syntaxin4 and pET15b-SNAP23 was induced with 0.5mM IPTG as described above. The cell pellet from 1L of culture was resuspended in 20mL of Buffer B (25mM HEPES-KOH, pH 7.5, 0.4M KCl, 10% glycerol, 20mM imidazole and 2mM β -mercaptoethanol) containing 5mM benzamidine, 1mM PMSF, 1x PIC, and subject to two passes through a French Press at 900 psi. Following detergent treatment and ultracentrifugation (as above), the supernatant was applied to 3mL of Ni-NTA resin (Qiagen) pre-equilibrated with Buffer B containing 100mM β -OG, and nutated for 2 h at 4°C. Resins were then washed with 30mL of Buffer B containing 100mM β -OG. Proteins were eluted in 25mM HEPES-KOH, pH 7.5, 0.4M KCl, 10% glycerol, 200mM imidazole and 100mM β -OG.

To purify MBP-Munc18a, Rosetta2/pMBP-TCS-Munc18a was grown in 1L of TB at 37°C and induced with 0.2mM IPTG at $OD_{600} \approx 1.0$. The culture was subsequently incubated at 22°C overnight before cell pellets were harvested by centrifugation (5k rpm, 5 min, RT, GS3 rotor). Cell pellet was resuspended in 20mL of Buffer C (50mM Tris-Cl, pH 8.0, 500mM KCl, 5mM EDTA) containing 5mM benzamidine, 1mM PMSF and 1x PIC, and then passed twice through a French Press at 900 psi. The Supernatants were collected through ultracentrifugation with a Beckman Type 70 Ti rotor (4°C, 1 hr, 40,000 rpm) and applied to 4mL of amylose resin pre-equilibrated with Buffer C. Following nutation at 4°C for 2 h, the amylose resin was washed with 20mL of Buffer C, then eluted with 20mL of Buffer C containing 10mM maltose. MBP-Munc18a was dialyzed 1,000,000-fold in RB150 (20mM HEPES-NaOH, pH 7.4, 150mM NaCl, 10% glycerol) overnight at 4°C. His₆-Munc18b and His₆-Munc18c were affinity-purified using Ni-NTA resin from lysates of transfected Sf9 cells (gift from Fengwei Bai) according to the published procedures [36]. His₆-Munc18b was dialyzed 27783-fold in RB150, and His₆-Munc18c was dialyzed 27783-fold into RB150 containing 0.5mM DTT. Proteins were concentrated to desired concentration using 30k MWCO Microsep™ Advanced Centrifugal Device (Pall Corporation) before storage.

Recombinant His₆-NSF, His₆- α SNAP and His₆-tev were each expressed in Rosetta2(DE3) in 1L of Terrific Broth at 37°C. Following addition of 0.5mM IPTG at $OD_{600} = 1.2$, the cultures were incubated for 4 h before cell pellets were harvested by centrifugation. His₆-NSF cell pellets were resuspended in 20mL of Buffer D (50mM HEPES-KOH, pH 7.6, 100mM KCl, 0.5mM MgCl₂, 0.5mM ATP, 5% glycerol, 1mM DTT) containing 10mM imidazole and 1x PIC, whereas His₆- α SNAP and His₆-Tev cell pellets were resuspended in 20mL of Buffer A containing 10mM imidazole and 1x PIC. Follow French Press and ultracentrifugation (as above), supernatants were applied to 4mL of Ni-NTA resin (pre-equilibrated with Buffer D or A, each containing 10mM imidazole), and nutated for 2 h at 4°C. After the resins were washed with

20mL of Buffer D or Buffer A (each containing 20mM imidazole), proteins were eluted in the respective buffers that contain 200mM imidazole. Prior to storage, His₆- α SNAP and His₆-Tev were dialyzed 1,000,000-fold in RB150 overnight at 4°C, whereas His₆-NSF was concentrated using 30k MWCO Microsep™ Advanced Centrifugal Device.

Proteoliposome Preparation

All the fluorescent lipids were obtained from Invitrogen whereas the non-fluorescent lipids were from Avanti Polar Lipids, Inc. Unless otherwise specified, donor proteoliposomes contain 60% POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), 17% POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine), 10% DOPS (1,2-dioleoyl-sn-glycero-3-phosphoserine), 10% cholesterol, 1.5% NBD-DHPE [N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine] and 1.5% rhodamine DHPE (Lissamine™ Rhodamine B 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine), and acceptor proteoliposomes contain 60% POPC, 19% POPE, 10% DOPS or POPS, 10% cholesterol and 1% Dansyl DHPE [N-(5-Dimethylaminonaphthalene-1-Sulfonyl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine]. Proteoliposomes were prepared by detergent dilution and isolated on a Histodenz density gradient flotation as previously described [38]. SNARE proteins were kept at similar densities as other reconstitution studies [26], with protein: lipid ratios at or below 1:200 for R-SNARE-bearing donor RPLs (reconstituted proteoliposomes) and at or below 1:500 for Q-SNARE-bearing acceptor RPLs. His₆-tev was added at 60 μ g/mL in each reconstitution to remove the N-terminal tags [38].

Lipid-Mixing Assay

Unless otherwise specified, a standard fusion reaction (20 μ L) contained R-SNARE donor RPLs (50 μ M lipids) and Q-SNARE acceptor RPLs (400 μ M lipids) in RB150. Reactions performed in the presence of NSF/ α SNAP also included 0.5mM MgCl₂, 0.5mM ATP, and an ATP regenerating system (0.5mg/mL creatine kinase and 14.5mM creatine phosphate). Wherever Munc18s were used, the N-terminal MBP or His₆ tag was removed by premixing the chimeric Munc18s with His₆-Tev at a molar ratio of 2:1. To monitor lipid mixing, reaction mixtures (prepared on ice, incubated overnight at 4°C or on ice) were transferred to a 396 well plate and the NBD fluorescent signal was measured (λ_{ex} = 460 nm, λ_{em} = 538 nm, λ_{cutoff} = 515 nm) in a Spectra-MAX Gemini XPS plate reader (Molecular Devices) at 37°C. The maximal, early rate of dequenching was calculated as the increased fluorescence at any time divided by the fluorescence at the first minute [(F_t - F₀)/F₀]. An increase of 1 in this parameter is defined as one unit. For donor RPLs, the dequenching [(F_d / F₀) - 1] in the presence of 2% (v/v) Triton X-100 was around 6 units in this study. To compare two sets of data, dequenching units from multiple repeats of each experimental condition were imported pair-wise into KaleidaGraph 3.6, where p values were calculated using Student's t test.

Results

Seven SNAREs Implicated in Mast Cell Exocytosis Form Multiple Fusogenic Trans-SNARE Complexes

In a systematic effort to identify the putative trans-SNARE complexes in mast cell exocytosis, we purified all seven SNAREs (VAMP2, 3, 7, 8, syntaxin3, 4, and SNAP-23) that are either biochemically or functionally implicated in the degranulation process. As shown in Fig 1A, syntaxin3 and syntaxin4 (lanes 5 and 6) were incorporated along with SNAP-23 into the acceptor proteoliposomes (without NBD-DPPE or Rh-DPPE), whereas comparable amounts of

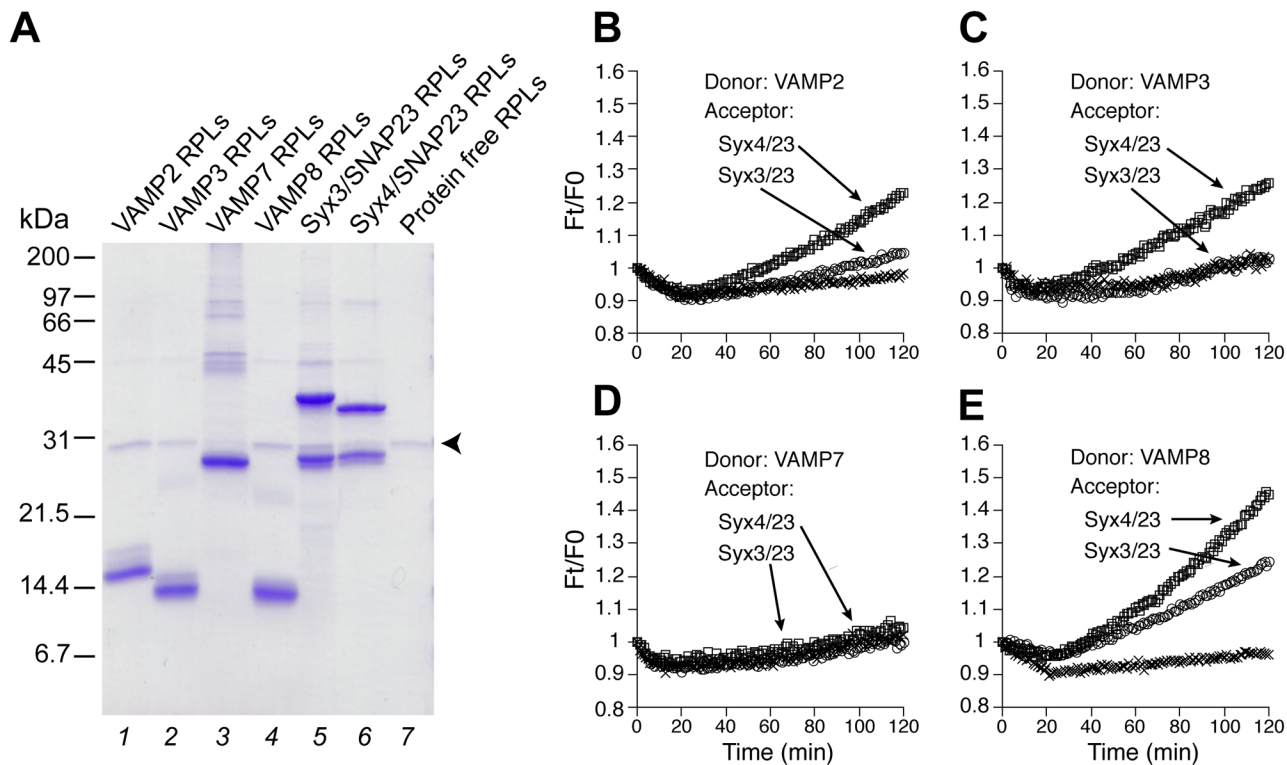


Fig 1. Reconstituted proteoliposomes (RPLs) bearing R- and Q-SNAREs involved in mast cell exocytosis. (A) Coomassie blue-stained SDS-PAGE of reconstituted proteoliposomes. A total of 20 nmol (based on total lipids) of donor RPLs (lanes 1 to 4) and acceptor RPLs (lanes 5 and 6) were used in each lane. Small amounts of His₆-Tev used in the reconstitution get incorporated as well (specified by the arrowhead). The positions of protein markers are indicated on the left. (B to E) Standard fusion reactions. The fluorescence of NBD-DHPE reconstituted in the donor RPLs was measured every min and the dequenching of NBD-DHPE fluorescence (due to lipid mixing) is presented as Ft/F₀, with Ft being the NBD-DHPE fluorescence at any time point and F₀ being the fluorescence at the first minute. Represented by x are controls (not readily visible in C and D), in which donor RPLs were incubated with the SNARE-free acceptor RPLs. A representative result from more than three biological replicates is shown.

doi:10.1371/journal.pone.0138683.g001

VAMP2, 3, 7, 8 (lanes 1 to 4) were incorporated into the donor proteoliposomes, with NBD-DPPE and Rh-DPPE at quenching concentrations. Upon fusion, the mixture of the donor and acceptor membranes relieves this quenching effect via dilution, leading to increased NBD fluorescence [39]. The fluorescent signal was recorded in a plate reader every minute (Fig 1B to 1E). The maximal, early rate of dequenching was calculated (see Materials and Methods) to more effectively compare lipid mixing from different reaction conditions (Fig 2). In our standard assay, low but detectable levels of lipid mixing were observed in six combinations (Figs 1B to 1E and 2, lanes 1 to 3): i) VAMP2/syntaxin3/SNAP-23, ii) VAMP2/syntaxin4/SNAP-23, iii) VAMP3/syntaxin3/SNAP-23, iv) VAMP3/syntaxin4/SNAP-23, v) VAMP8/syntaxin3/SNAP-23, and vi) VAMP8/syntaxin4/SNAP-23. VAMP8 appears to be the most potent R-SNARE whereas VAMP7 shows no activity.

In eukaryotic cells, trans-SNARE pairing is facilitated by tethering factors (e.g., HOPS for vacuole fusion and the exocyst for neurotransmission), which bring vesicles/membranes into close proximity. This can be mimicked *in vitro* by increasing the concentrations of SNARE-bearing liposomes or by introducing synthetic polymer polyethylene glycol (e.g., PEG6000) [38,40]. Enhancing the concentrations of SNARE-bearing liposomes enhances the rate of the specific interaction between cognate SNAREs located on the donor and the acceptor, promoting their tethering and docking. PEG on the other hand induces nonspecific membrane tethering, which has been exploited extensively in previous studies of reconstituted SNARE-bearing

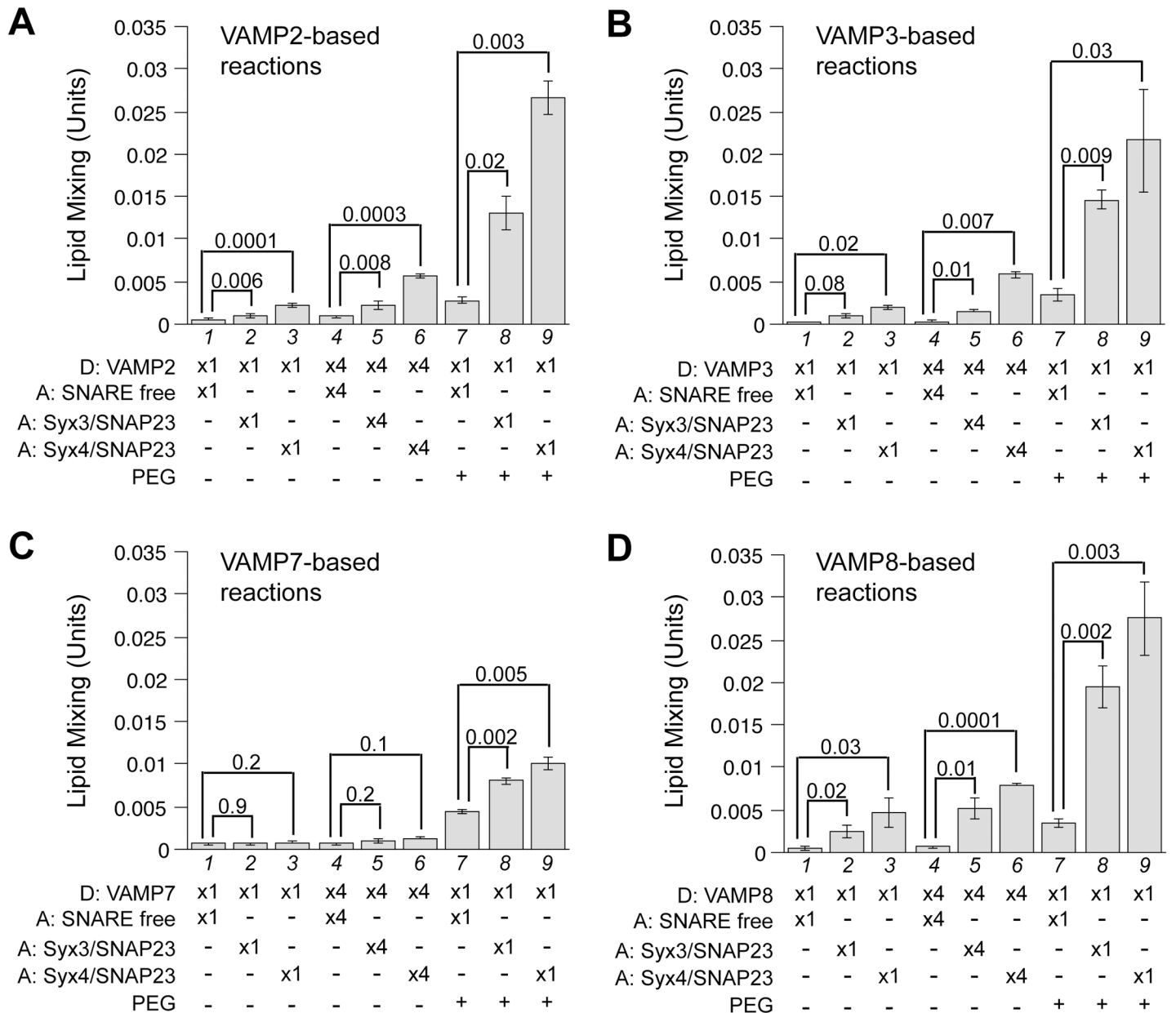


Fig 2. Functional pairing of SNAREs examined under enhanced tethering conditions. Donor RPLs bearing VAMP2 (A), VAMP3 (B), VAMP7 (C) or VAMP8 (D) were incubated with acceptor RPLs in standard fusion reactions (lanes 1 to 3), 4 x reactions (lanes 4 to 6), where the concentrations of the donor and acceptor RPLs were increased 4 fold to 200mM and 1600mM respectively. Fusion reactions including 4% PEG6000 (v/v) are presented in lanes 7 to 9. The maximal early rates of dequenching/lipid mixing were calculated as described in Materials and Methods. The mean values are presented and error bars represent standard deviations from at least three independent experiments. Where appropriate, p values calculated using Student's t test are presented.

doi:10.1371/journal.pone.0138683.g002

proteoliposomes [41,42]. As shown in Fig 2, except for VAMP7, a 4-fold increase of both donor and acceptor liposomes modestly increased the rates of SNARE-mediated lipid mixing (compare lanes 2 and 5, 3 and 6). More drastic effects were observed when PEG was administered (lanes 8 and 9). Notably, the lipid mixing mediated by VAMP2/syntaxin3/SNAP-23 or VAMP3/syntaxin3/SNAP-23 that is almost negligible under standard reaction conditions now becomes readily detectable (Fig 2A and 2B, compare lanes 8 and 2). Even for VAMP7, Q-SNARE-dependent lipid mixing can be observed relative to the control (Fig 2C, compare

lanes 8 and 9 to 7). These data indicate that an accurate assessment of trans-SNARE pairing in reconstitution may require conditions where the efficiency of tethering is optimized.

Recapitulated SNARE-Dependent Mast Cell Exocytic Fusion Is Sensitive to SNAP and NSF

We went on to examine the nature of lipid mixing by introducing SNARE disassembly chaperones (α SNAP and NSF) into all except the VAMP7-based reactions. This is because the subdued VAMP7 activity—likely due to its inhibitory N-terminal longin domain [43]—prevents accurate assessment of α SNAP /NSF-dependent inhibition. Even in the presence of PEG, the fold change between the signal (Fig 2C, lanes 8 and 9) and the background (lane 7) would be too small for such investigation. For the other R-SNAREs (e.g., VAMP2, VAMP3, and VAMP8), the addition of α SNAP at high concentrations (S1 Fig) clearly inhibits reconstituted lipid-mixing reactions, which reflects α SNAP's ability to prevent membrane fusion by binding to individual SNAREs, its *bona fide* receptors, or to the partially assembled trans-SNARE complex, as observed in other membrane fusion systems [44,45,46]. At non- or sub-inhibitory concentrations of α SNAP, addition of NSF, an ATPase, diminished the rates of lipid mixing (Fig 3). NSF by itself had minimal effect on most fusion reactions (Fig 3, compare lanes 1 and 6). Therefore, the cooperation of α SNAP and NSF is required to disassemble either the trans-SNARE complexes or the Q-SNARE subcomplexes prior to trans-SNARE zippering. Taken together, we conclude that all six sets of fusion reactions reconstituted in this study are underpinned by authentic trans-SNARE interactions.

Munc18a Promotes the Lipid Mixing Mediated by Four Sets of SNAREs

To investigate if Munc18a, b, and c operate through any of the trans-SNARE complexes we have identified, we purified recombinant forms of these proteins either from *E. coli* lysates or from cultured Sf9 cells (recombinant Munc18b and Munc18c expressed in *E. coli* are largely insoluble and difficult to purify). The N-terminal tags are readily removed by Tev protease (S2 Fig). When we initially tested Munc18a in lipid-mixing assays, it potently stimulated the rate of VAMP2-mediated lipid mixing in a concentration-dependent fashion (S3 Fig). At the suboptimal level (e.g., 2 μ M), it stimulated the lipid mixing mediated by VAMP2 (Fig 4, lanes 1 to 3), VAMP3 (lanes 4 to 6), but not by VAMP7 (lanes 7 to 9) or VAMP8 (lanes 10 to 12). The specificity of Munc18a for VAMP2 mirrors what had been previously observed in SNAP-25-mediated neurotransmission, where selectively replacing VAMP2 residues in the SNARE domain with corresponding residues in VAMP8 decreased Munc18a stimulation *in vitro* and reduced exocytosis *in vivo* [26]. Munc18a does not have any effect unless acceptor liposomes bear either syntaxin3/SNAP-23 or syntaxin4/SNAP-23, suggesting the presence of cognate Q-SNAREs on the apposing membrane is important for Munc18a action.

Intriguingly, neither Munc18b nor Munc18c exhibits positive effects in the fusion reactions (S4A and S4B Fig) as we had expected. The pull-down assay shows that Munc18c interacted with full-length syntaxin4 but not syntaxin3 (S4C Fig), which corroborates with published studies [31,47]. This interaction might be necessary for Munc18c-dependent inhibition of lipid mixing catalyzed by VAMP8/syntaxin4/SNAP-23 (S4B Fig, lane 12; S5D Fig), but does not appear to be the exclusive underlying mechanism since VAMP3-mediated lipid mixing was not negatively affected (S4B Fig, lane 6). In contrast to Munc18c, Munc18b had little effect on virtually all combinations tested. In addition to its reported ability to bind directly to syntaxin3 cytoplasmic domain [47], Munc18b also appears to bind membrane-anchored full-length syntaxin3 and syntaxin4 (S4C Fig). The significance of Munc18b/syntaxin4 interaction in mast cell exocytosis and membrane fusion requires future investigation.

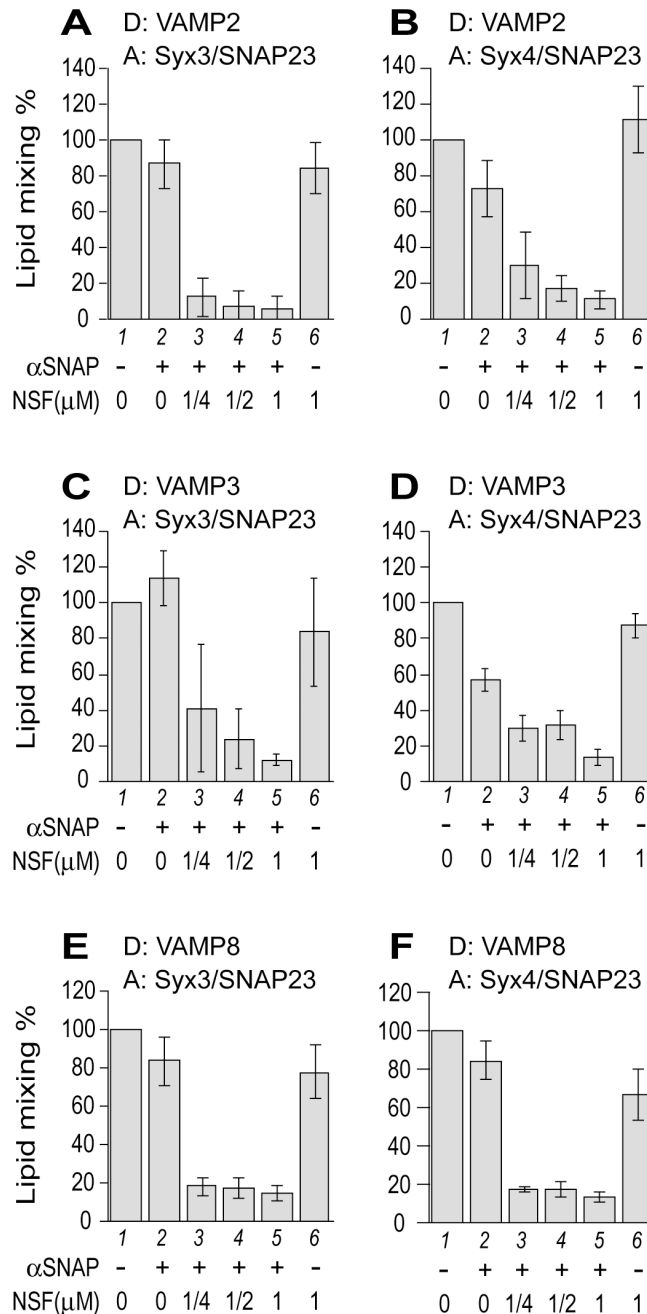


Fig 3. αSNAP/NSF-dependent inhibition of SNARE-mediated lipid mixing. (A and B) VAMP2-bearing donor RPLs were incubated with specified acceptor RPLs in fusion reactions containing 0.5mM ATP, 0.5mM MgCl₂, and 4% PEG6000. Also included in the reactions are specified amounts of NSF along with 0.5μM αSNAP (A) or 0.15μM αSNAP (B). (C and D) VAMP3-bearing donor RPLs were incubated with specified acceptor RPLs in fusion reactions containing 1mM ATP, 1mM MgCl₂, and 4% PEG6000. Also included in the reactions are specified amounts of NSF along with 0.15μM αSNAP. (E and F) VAMP8-bearing donor RPLs were incubated with specified acceptor RPLs in standard reactions containing 0.5mM ATP and 0.5mM MgCl₂. Also included in the reactions are specified amounts of NSF along with 0.1μM αSNAP (E) or 0.02μM αSNAP (F). All samples contained the same amounts of αSNAP buffer and NSF buffer. The maximal early rates of lipid mixing for the SNARE-only reactions were used to generate the “standard” value (the lipid-mixing rate from SNARE-free RPLs was treated as a background and subtracted) and set as 100%. The values for other conditions were adjusted relative to the “standard” value. Error bars represent standard deviations from three independent experiments.

doi:10.1371/journal.pone.0138683.g003

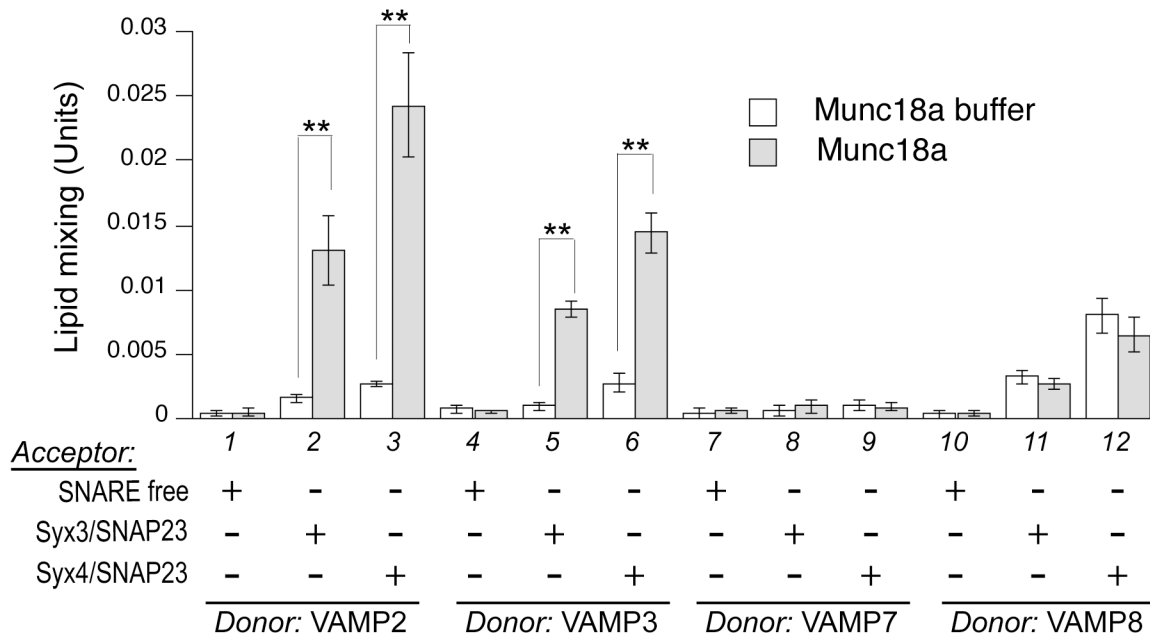


Fig 4. Munc18a selectively regulates different trans-SNARE complexes. Various combinations of donor and acceptor RPLs as specified were incubated overnight at 4°C with Munc18a (2µM) or control buffer, before transferring to 37°C. Error bars represent standard deviations from three independent experiments. p values were calculated using Student's t test. ** indicates p < 0.01.

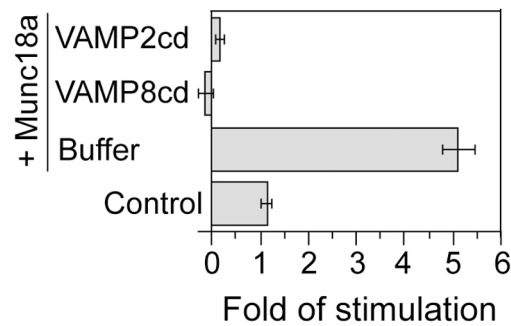
doi:10.1371/journal.pone.0138683.g004

Because Munc18s could regulate exocytosis via reversible interaction with the N-peptide in cognate syntaxins [48,49], we wished to address the concern whether the tetrapeptide (GAMG) remaining at the N-terminus of syntaxin4 after Tev cleavage might interfere with Munc18 function. We acquired an untagged syntaxin4 construct and co-expressed it along with His₆-SNAP-23 as previously described [36]. Acceptor RPLs bearing untagged syntaxin4 behaved similarly to tagged syntaxin4, in their response to Munc18 isoforms (S5 Fig), suggesting that the extra N-terminal tetrapeptide had caused minimal impact in our assay. Based on reported studies in other secretory events [34,50], we propose that Munc18b or Munc18c might receive post-translational modifications in activated mast cells in order to promote SNARE-dependent granule exocytosis (see discussion).

Munc18a-Dependent Stimulation Is Sensitive to Soluble Fragments of R-SNAREs at an Early Stage

To further characterize the synergistic effect between Munc18a and VAMP2/syntaxin4/SNAP-23 in lipid mixing, we introduced the cytoplasmic domains of VAMP8 and VAMP2 at a concentration 8 fold of the full-length VAMP2 on the donor liposomes. These inhibitory proteins prevent SNARE-dependent fusion by competing for cognate Q-SNAREs [19,51]. When they were incubated with RPLs overnight on ice before the addition of Munc18a, very little lipid-mixing activities were observed (Fig 5A), showing that Munc18a-promoted lipid mixing requires functional trans-SNARE pairing. Intriguingly, when these inhibitory proteins were introduced to the reaction mixture 90 min after Munc18a addition, and the mixture was then incubated on ice overnight, there was very little inhibition (Fig 5B). These observations indicate that VAMP8cd or VAMP2cd does not poison the lipid-mixing reaction in any unspecific fashion. Rather, they act in a particular stage in the fusion cascade that is kinetically earlier than Munc18a action. We suggest that Munc18a either promotes the partial zippering of the trans-

A V2cd/V8cd added before Munc18a



B V2cd/V8cd added after Munc18a

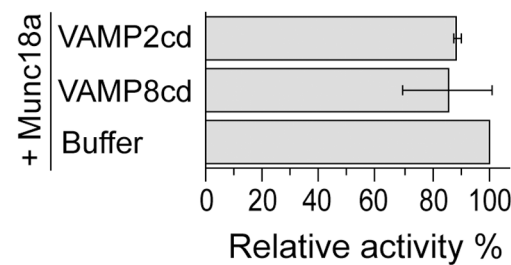


Fig 5. Munc18a-dependent stimulation is sensitive to inhibitory proteins at an early stage of the fusion reaction. (A). Acceptor RPLs bearing untagged syntaxin4/His₆-SNAP-23 and VAMP2-bearing donor RPLs were incubated with inhibitory proteins VAMP2cd (2μM) or VAMP8cd (2μM) or buffer on ice overnight and then received 5μM Munc18a or MBP (control). The incubation was continued on ice for another 90 min before shifting to 37°C. Fold increases in the initial lipid-mixing rates of the fusion reactions are shown. In (B), the same RPLs were incubated first with 5μM Munc18a on ice for 90 min before the addition of VAMP2cd (2μM) or VAMP8cd (2μM). Following overnight incubation on ice, samples were transferred to 37°C to monitor NBD fluorescence. The maximal early rates of lipid mixing for the Munc18a-only reactions were used to generate the “standard” value and set as 100%. The values for other conditions were adjusted relative to the “standard” value. Error bars represent standard deviations from three independent experiments.

doi:10.1371/journal.pone.0138683.g005

SNARE complex on ice, which becomes inaccessible to VAMP2cd or VAMP8cd, or the binding of Munc18a to the SNAREs prevents the access of the inhibitory proteins. Future studies are needed to distinguish these scenarios.

Discussion

Identification of functional trans-SNARE complexes in mast cell exocytosis is often challenged by the presence of both compound degranulation and piecemeal degranulation, which can be further compounded by the heterogeneity of granule population inside the cell [7,15]. Nevertheless, in addition to VAMP8/syntaxin4/SNAP-23, biochemical characterizations, cellular localization studies, and cell-based functional analysis have collectively indicated the requirement of syntaxin3-based trans-SNARE complexes in mast cell exocytosis [9,16,20,52]. Our reconstitution of several fusogenic syntaxin3-containing trans-SNARE complexes under enhanced tethering conditions lends strong support to the notion that distinct trans-SNARE complexes underscore various types of granule/lysosome exocytosis in mast cells.

All the SNARE-only fusion reactions tested in this study are sensitive to action of ATPase NSF and its cofactor αSNAP. This is consistent with the reported behavior of proteoliposomes

bearing vacuolar SNAREs, which do not fuse at all in the presence of Sec17p (α SNAP homolog in yeast) and Sec18p (NSF homolog in yeast) unless the HOPS tethering complex is also present [38] [53]. Intriguingly, the reconstituted synaptic vesicle fusion appears to resist the disassembly effects of α SNAP and NSF [54]. In the absence of a self-inhibitory domain of syntaxin1A, the addition of α SNAP and NSF could even accelerate the lipid mixing. It is conceivable that, due to the differences in the SNARE complexes or the different lipid composition in different proteoliposome fusion systems, the neuronal trans-SNARE complex executes fusion at rate faster than the rate of turnover by α SNAP and NSF [53].

How could Munc18a promote β -hexosaminidase release [30] if it does not operate through VAMP8-based trans-SNARE complexes (Fig 4, lanes 11 and 12)? Our observation that Munc18a stimulates VAMP2- and VAMP3- dependent lipid mixing implies that a distinct set of trans-SNARE complex might form in the event that VAMP8-dependent degranulation is compromised. In agreement to this, a ternary complex of VAMP2, syntaxin4 and SNAP-23 was identified in the lipid rafts during mast cell exocytosis [55], and more importantly, increasing amounts of VAMP2 and VAMP3 were found in association with SNAP-23 in VAMP8--knockout cells [7,8]. Similar compensatory mechanisms have previously been observed in intracellular traffic. In baker's yeast, a predominantly Golgi-localized SNARE Ykt6p is up-regulated in *sec22* deletion strains to sustain ER to Golgi traffic [56] and in *nyv1* deletion strains to sustain vacuolar fusion [57]. In animal cells, VAMP3 can partially compensate for the deletion of VAMP2 in calcium-triggered exocytosis [58,59]. Although blocking or knocking down VAMP2 or VAMP3 alone had limited effects on β -hexosaminidase release [10,19], we hypothesize that simultaneously knocking out VAMP2, VAMP3, and VAMP8 would abolish mast cell exocytosis.

Concerning the activators for VAMP8-based degranulation, Munc18 isoforms remain the top candidates. Although none of the three unmodified Munc18s operate synergistically with VAMP8-based trans-SNARE complexes in this study, a number of reports have documented the importance of reversible phosphorylation in Munc18 activity. For example, site-specific phosphorylation of Munc18a by PKC has been found critical for neurotransmission [60]. Since the PKC pathway is also active in mast cell degranulation [61,62,63], it will be interesting to examine if the same Munc18a modifications also takes place and whether they alter the specificity of Munc18a. Similarly, polarized secretion in epithelial cells requires CDK5-dependent phosphorylation of Munc18b at Thr572, which promotes the assembly of the functional Munc18b/VAMP2/syntaxin3/SNAP-25 membrane fusion machinery [50]. For GLUT4 exocytosis in fat and muscle cells, Munc18c phosphorylation at Y521 promotes SNARE complex formation between VAMP2, syntaxin4, and SNAP-23 [34], whereas the unmodified Munc18c inhibits membrane fusion via specific interaction with syntaxin4 [34,35]. Future studies of signaling-dependent modifications of Munc18 in activated mast cells will provide new insights into the selective regulation of degranulation-relevant trans-SNARE complexes.

Supporting Information

S1 Fig. Effects of α SNAP on SNARE-only reactions.

(EPS)

S2 Fig. Purified Munc18s are TEV cleavable.

(EPS)

S3 Fig. Munc18a stimulates VAMP2-mediated lipid mixing in a concentration dependent fashion.

(EPS)

S4 Fig. The effects of Munc18b and Munc18c in reconstituted fusion reactions.
(EPS)

S5 Fig. The extra tetrapeptide at the N-terminus of the recombinant syntaxin4 does not alter the selective activities of Munc18s.
(EPS)

S1 File. Supplemental Figure Legends.
(DOCX)

S1 Table. Oligonucleotide primers used in PCR for cloning.
(PDF)

Acknowledgments

We are grateful to Drs William Trimble, Ulrich Blank, Thomas Südhof, Paul Roche, Reinhard Jahn, Jose Rizo, Jingshi Shen, Fengwei Bai, and William Wickner for reagents. We thank Mississippi-IDeA Network of Biomedical Research Excellence for the use of the core facility, and Drs. Gordon Cannon and Sabine Heinhorst for the use of the French Press equipment.

Author Contributions

Conceived and designed the experiments: HX. Performed the experiments: HX MGA SVK. Analyzed the data: HX MGA SVK. Contributed reagents/materials/analysis tools: HX MGA SVK. Wrote the paper: HX MGA.

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