The Neurexin/N-Ethylmaleimide-sensitive Factor (NSF) Interaction Regulates Short Term Synaptic Depression**

Received for publication, February 9, 2015, and in revised form, April 24, 2015 Published, JBC Papers in Press, May 7, 2015, DOI 10.1074/jbc.M115.644583

Tao Li‡, Yao Tian†, Qian Li†, Huiying Chen‡, Huihui Lv‡, Wei Xie§§, and Junhai Han‡§

From the †Institute of Life Sciences, Key Laboratory of Developmental Genes and Human Disease, Southeast University, Nanjing 210096 and the ‡§Co-innovation Center of Neuroregeneration, Nantong University, Nantong, JS 226001, China

Background: Neurexins (NRXs) are cell adhesion molecules and regulate synapse formation and synaptic transmission.

Results: NRX associates with NSF in vivo, and the absence of NRX/NSF interaction results in abnormal distribution and impaired function of NSF.

Conclusion: NRX/NSF interaction facilitates NSF recruitment and subsequent SNARE complex disassembly.

Significance: Neurexin regulates the presynaptic exocytotic machinery.

Although Neurexins, which are cell adhesion molecules localized predominantly to the presynaptic terminals, are known to regulate synapse formation and synaptic transmission, their roles in the regulation of synaptic vesicle release during repetitive nerve stimulation are unknown. Here, we show that nrx mutant synapses exhibit rapid short term synaptic depression upon tetanic nerve stimulation. Moreover, we demonstrate that the intracellular region of NRX is essential for synaptic vesicle release upon tetanic nerve stimulation. Using a yeast two-hybrid screen, we find that the intracellular region of NRX interacts with N-ethylmaleimide-sensitive factor (NSF), an enzyme that mediates soluble NSF attachment protein receptor (SNARE) complex disassembly and plays an important role in synaptic vesicle release. We further map the binding sites of each molecule and demonstrate that the NRX/NSF interaction is critical for both the distribution of NSF at the presynaptic terminals and SNARE complex disassembly. Our results reveal a previously unknown role of NRX in the regulation of short term synaptic depression upon tetanic nerve stimulation and provide new mechanistic insights into the role of NRX in synaptic vesicle release.

Neurexins are cell adhesion molecules that are thought to be the key presynaptic organization molecules involved in synaptogenesis, synaptic transmission, and synapse maintenance (1–6). The extracellular region of the NRX molecule binds to Neuroligins (7, 8), Dystroglycan (9), Neurexophilin (10–12), leucine-rich repeat proteins (LRRTM2) (13, 14), and Cerebellin (15). In contrast, the intracellular region of the NRX molecule associates with several molecules involved in the synaptic vesicle exocytosis machinery, including synaptotagmin (16) and the PDZ domain-containing proteins CASK (17), Mints (18), and syntenin (19). The neuroligins are localized to the postsynaptic densities (20) and are associated with the neurotransmitter receptors (21, 22). The trans-synaptic interaction of NRX and Neuroligin therefore bridges the synaptic cleft to align the pre- and postsynaptic neurotransmitter release machinery with the postsynaptic neurotransmitter receptors. Previous studies have shown that the α-NRXs functionally couple Ca2+ channels to the presynaptic machinery to mediate synaptic vesicle exocytosis (6). The loss of the single α-NRX in Drosophila leads to impaired evoked synaptic transmission and reduced quantal content (4).

Neurotransmitter release is mediated by the fusion of synaptic vesicles, which is triggered by Ca2++ and executed by soluble NSF2 attachment protein receptors (SNAREs). During fusion, vesicular and target SNAREs assemble into an α-helical trans-SNARE complex that forces the two membranes together to form a highly stable SDS-resistant 7S SNARE complex (24–26). After fusion, soluble NSF attachment proteins (SNAPs) bind to SNAREs and then mediate the binding with the ATPase NSF (25), which mediates the disassembly of SNARE complexes and regenerates free SNAREs to be used in subsequent fusion reactions (27). Live image studies have shown that NSF mutant (i.e. cont) synapses exhibit defective NSF re-distribution during tetanic nerve stimulation (28), suggesting some potential mechanisms to restrict the mobilization of NSF. However, the molecular mechanism to restrict the mobilization of NSF is not clear.

Here, we show that the Drosophila homolog of α-NRX interacts with NSF at the presynaptic terminals. The absence of this interaction in nrx mutant synapse leads to rapid short term synaptic depression during tetanic nerve stimulation. We further demonstrate that the NRX/NSF interaction facilitates NSF recruitment to the presynaptic terminals and promotes SNARE complex disassembly. Our results therefore reveal a novel role of NRX in the regulation of short term synaptic depression and an unknown linkage between NRX and the presynaptic exocytotic machinery.

* This work was supported by Ministry of Science and Technology Grants 2014CB942803 and 2012CB517903, Research Project of Chinese Ministry of Education Grant 113028A, Excellent Youth Foundation of Jiangsu Province of China Grant BK20140024 (to J. H.), and National Natural Science Foundation of China Key Program 30930051 (to W. X.). The authors declare that they have no conflicts of interest with the contents of this article.

† To whom correspondence should be addressed: Institute of Life Sciences, Southeast University, 2 Sipailou Rd., Nanjing 210096, China. Tel.: 86-25-83790962; Fax: 86-25-83790962; E-mail: junhaihan@seu.edu.cn.

‡ This article was selected as a Paper of the Week.

1 The abbreviations used are: NSF, N-ethylmaleimide-sensitive factor; EJC, excitatory junction current; MBP, maltose-binding protein; NMJ, neuromuscular junction.
Experimental Procedures

Fly Genetics—The flies were maintained on a standard medium at 25 °C with 60–80% relative humidity. The wild-type flies used in this study were w1118. The α-nrx (CG7050) null mutant allele, nrx273, was obtained from Dr. Manzoor A. Bhat’s laboratory (4). The other α-nrx null mutant allele, nrxΔIII, was generated by p-element imprecise excision using Δ2–3 as a transposase source according to standard procedures. The detailed experimental process has been described in a previous report (3). To eliminate potential genetic background effects, the recombinant of two out-crossed nrx null mutant alleles, nrxΔIII/nrx273, was used in this study. To generate the NRX transgenes, the full-length NRX cDNAs and the cDNAs that encode either the C-terminally truncated NRXAC or the PDZ-binding motif-deleted NRXΔ4 were subcloned into the pUAST vector and injected into w1118 flies.

Antibodies—The anti-NSF antibody was generated against a purified GST-NSF (amino acids 1–746) fusion protein in rabbits. An affinity column, which was created by coupling MBP-NSF fusion protein to CNBr-activated Sepharose 4B, was used to purify the antibody. The anti-NRX and anti-Ecp antibodies were generated as described previously (29, 30). Other antibodies were obtained from Developmental Studies Hybridoma Bank (syntaxin, 6C3; DLG, 4F3; and BRP, nc82) and GenScript (mouse His tag antibody and rabbit GST tag antibody).

Electrophysiology—Third instar larvae were carefully dissected as described previously (31). Two-electrode voltage clamp recordings were performed with an Axoclamp 900A amplifier (Molecular Devices, Sunnyvale, CA) using both recording (15–20 megohms) and current-passing (5–8 megohms) electrodes filled with 3 M KCl. The EJCs were recorded from ventral longitudinal muscles 6/7 in abdominal segments A2/A3 at a holding potential of −60 mV. Unless indicated otherwise, all recordings were conducted in a standard solution containing 110 mM NaCl, 5 mM KCl, 4 mM MgCl2, 1.8 mM CaCl2, 36 mM sucrose, and 5 mM HEPES, pH 7.0. Data were acquired using Axoclamp 900A software and Clampex 10.2 software. Synaptic currents were sampled at 10 kHz and low-pass filtered at 1 kHz. Analysis of synaptic current data was carried out in the Mini Analysis Program.

Yeast Two-hybrid Screen—The yeast two-hybrid screening was performed according to the MatchMaker™ Gold Yeast Two-Hybrid System User Manual (Clontech). Briefly, the C terminus of NRX (amino acids 1,716–1,838) was expressed as a bait in the yeast strain Y2HGold. The bait strains were incubated with prey strains Y187 that contained Drosophila cDNA libraries (Clontech) to generate zygotes. The zygotes then were screened on double dropout media containing X-α-Gal and aureobasidin A and further confirmed on quadruple dropout media containing X-α-Gal and aureobasidin A. The plasmids from the positive clones were sequenced and identified using the BLAST program and the FlyBase database.

Co-immunoprecipitation—Fly heads (n = 200) were homogenized on ice in 500 μl of PBS with a protease inhibitor containing 1% CHAPS. After 30 min of rotation at 4 °C, the extracts were centrifuged at 16,000 × g for 5 min at 4 °C. Next, 200 μl of the supernatant was diluted with 1 ml of PBS containing a protease inhibitor, after which either 20 μl of the NRX antibody or 1 μl of the preimmune serum (as a negative control) was added and incubated for 2 h at 4 °C. After blocking with 2% BSA in PBS buffer for 30 min at 4 °C, 50 μl of protein A beads (Sigma) were added to the tubes and incubated for 1 h at 4 °C. After three washes with PBS containing 0.2% CHAPS, the immune complexes were eluted with 2× SDS sample buffer and subjected to SDS-PAGE and Western blotting.

Subcellular Membrane Fraction—The subcellular membrane fractions were separated using glycerol velocity sedimentation as described previously (32). Briefly, 3 g of flies were decapitated in liquid nitrogen and ground to a powder in a mortar and pestle. The powdered heads were resuspended in 300 μl of lysis buffer (150 mM NaCl, 10 mM HEPES, pH 7.4, 1 mM EGTA) and homogenized in a Dounce pestle. The postnuclear supernatant (10 min at 1,000 × g) was layered carefully onto a 5–25% glycerol gradient made in lysis buffer over a 50% sucrose cushion. The gradients were centrifuged for 1 h at 45,000 rpm in an SW60Ti rotor (Beckman, Brea, CA). All steps were performed at 4 °C. The gradient fractions then were subjected to SDS-PAGE and Western blotting.

Pulldown Assay—All GST fusion proteins, His tags proteins, and MBP fusion proteins were expressed in Escherichia coli BL21 cells and purified with glutathione-Sepharose (GE Healthcare, Little Chalfont, Buckinghamshire, UK), nickel-nitritotriacetic acid-agarose (Qiagen, Hilden, Germany), and amylose resin (New England Biolabs, Ipswich, MA), respectively. To map the binding site in NRX, MBP-NSF fusion protein-coupled beads were incubated with various purified GST-NRX fragments (~20 μg of purified GST-NRX fragments each). To map the binding site in NSF, GST-NSF fusion protein-coupled beads were incubated with 40 μg of purified His-tagged C-terminal NRX fusion protein (His6-NRX). After washing, the elution was analyzed by Western blotting.

Binding Affinity Assay—1.2 μg of GST-NSF fusion proteins were coupled to glutathione-Sepharose resins and incubated with various concentrations of purified His6-NRX at 4 °C for overnight. For the incubations, no extra Ca2+ or EGTA was added. After three washes, the elution was subjected to SDS-PAGE and Western blotting.

For Ca2+-dependent binding assay, GST-NRX-C-terminal fusion protein-coupled beads were incubated with the extracts of 200 fly heads (in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, and protease inhibitors) at indicated EDTA or Ca2+ concentrations. After washing, the elution was analyzed by Western blotting.

Immunostaining and Analysis—Immunostaining was performed as described previously (33). Briefly, third instar larvae were dissected, fixed, and then incubated with the primary antibodies, rabbit anti-NRX (1:30), rabbit anti-NSF (1:50), mouse anti-DLG (1:100), mouse anti-BRP (1:50), and goat anti-HRP (1:100) overnight. After four washes, the samples were incubated with secondary antibodies at room temperature for 2 h. After four washes, the samples were examined under an LSM 700 confocal microscope. The boutons in the ventral longitudinal muscles 6/7 of abdominal segment 3 were analyzed. To quantify the distribution of NSF, the inner circle of the DLG pattern was defined as the “presynaptic membrane.” We drew a

Neurexin Regulates Synaptic Depression
smaller inner circle 0.25 μm away from and along the presynaptic membrane and defined the area between the two circles as the "membrane." The intensity of the membrane NSF and total NSF was measured in each genotype, and the ratio of the membrane NSF to the total NSF was quantified and presented.

Preparation of 7S SNARE Complex—7S SNARE complexes were prepared as described previously (34). Briefly, 10 flies were collected in an EP tube and frozen in liquid nitrogen and vortexed, and heads were homogenized in 50 μl of SDS sample buffer on ice. After centrifugation to the pellet cuticle, 10 μl of supernatant were loaded onto SDS-polyacrylamide gels without boiling and electrophoresed at 15 mA per gel. The gels were immunoblotted with syntaxin antibody at 1:1,000 dilution.

Statistical Analysis—Data are presented as the mean ± S.E. For all statistical analyses, two-tailed Student’s t tests were used to compare genotypes. Significance was classified as follows: *, p ≤ 0.05; **, p < 0.01; ***, p < 0.001; not significant (n.s.), p > 0.05.

Results

nrx Mutant Synapses Show Normal Short Term Synaptic Depression during Low Frequent Repetitive Nerve Stimulation—Mammals possess three nrx genes, each of which transcribes α- or β-nrxs via two promoters (35, 36). In contrast, only one α-nrx gene has been discovered in Drosophila (3, 4). To assess the potential roles of NRX in the regulation of synaptic transmission during repetitive nerve stimulation, two-electrode voltage clamp analyses were performed to record the EJCs at the Drosophila neuromuscular junction (NMJ). When recordings were performed at 0.5 mM Ca2+ in both wild-type and nrx mutant synapses showed mild short term synaptic depression in response to 1 Hz stimulation (Fig. 1A). However, the current amplitudes in neurexin mutant synapses were significantly reduced compared with that of wild-type synapses (Fig. 1, A and B). Previous studies in mammals have suggested that the α-NRXs functionally couple Ca2+ channels to the presynaptic machinery to mediate synaptic vesicle exocytosis, and studies in fly have shown that abnormal Ca2+ sensitivity of neurotransmitter release in nrx mutant synapse was completely restored when recordings were performed at 1 mM Ca2+ (4, 6). Consistent with a previous report (4), an increase in the Ca2+ concentration restored the amplitude of EJCs at the NMJ (Fig. 1, C and D). To eliminate the effects of abnormal Ca2+ sensitivity in neurotransmitter release, we performed our recordings at 1.8 mM Ca2+. Under this condition, the nrx mutant synapses displayed mild short term synaptic depression in response to 1 Hz stimulation, which was similar to the response of the wild-type synapses (Fig. 1E).

nrx Mutant Synapses Exhibit Rapid Short Term Synaptic Depression during Tetanic Nerve Stimulation—We further performed two-electrode voltage clamp recordings to examine the EJCs at the Drosophila NMJ upon 40 Hz tetanic nerve stimulation. Interestingly, a marked activity-dependent reduction in the amplitude of the evoked currents was observed in the nrx mutant relative to the wild-type synapses under 0.5 mM Ca2+ conditions (Fig. 2, A and B). Rapid short term synaptic depression in nrx mutant synapses might be due to abnormal Ca2+ sensitivity and/or some other potential effectors. To eliminate the effects of abnormal Ca2+ sensitivity in short term synaptic

FIGURE 1. nrx mutant synapses show normal synaptic depression during low frequent repetitive nerve stimulation. A, 1 Hz stimulation for 200 s with 0.5 mM Ca2+ in wild-type (black, n = 8) and nrx mutant (gray, n = 10) synapses. B, mean EJC amplitudes in the wild-type (black, n = 8) and nrx mutant (gray, n = 10) synapses. C, example traces of evoked EJCs supplied with variant external Ca2+ concentration in the wild-type and nrx mutant synapses. D, mean EJC amplitudes in the wild-type (black, n = 8) and nrx mutant (gray, n = 8) synapses supplied with variant external Ca2+ concentration. E, 1 Hz stimulation for 200 s with 1.8 mM Ca2+ in wild-type (black, n = 5) and nrx mutant (gray, n = 5) synapses. F, mean steady-state EJCs with 1.8 mM Ca2+ in the wild-type (black, n = 8) and nrx mutant (gray, n = 8) synapses. G, 40 Hz tetanic stimulation with 0.5 mM Ca2+ in the wild-type and nrx mutant synapses. H, mean steady-state quantal content with 1.8 mM Ca2+ in the wild-type (black, n = 5) and nrx mutant (gray, n = 5) synapses.
depression, we further conducted the recordings at 1.8 mM Ca^{2+}. Under this condition, nrx mutant synapses still exhibit a rapid synaptic depression compared with wild-type synapses (Fig. 2, C and D). The amplitude of the evoked currents in the wild-type synapses quickly declined to 75% of the initial stimulus after eight stimuli (Fig. 2E) and to 50% after 6.0 ± 1.5 s and then slowly reached a steady state of 72.0 ± 11.6 nA (n = 10) after ~20 s (Fig. 2, C–F). In contrast, the amplitude of the evoked currents in the nrx mutant synapses dropped precipitously to ~62% of the initial stimulus after eight stimuli (Fig. 2E) and to 50% after 0.9 ± 0.4 s and reached a steady state of 40.5 ± 9.9 nA (n = 8) after ~15 s (Fig. 2, C–F). The rapid short term synaptic depression in the nrx mutant synapse is caused by the presynaptic loss of NRX, as the neural expression of NRX restored this deficit in the nrx mutant synapses (Fig. 2, C and D).

Short term synaptic depression during tetanic stimulation in the nrx mutant synapses may reflect either the rapid desensitization of postsynaptic glutamate receptors or deficits in synap-
Neurexin Regulates Synaptic Depression

By differentiating between these two possibilities, we compared the kinetics of the individual synaptic current at the initiate state and at the steady state of tetanic stimulation. In both wild-type and nrx mutant synapses, the decay time of the individual synaptic currents at the steady state were comparable with that at the initiate state of tetanic stimulation (Fig. 2, G and H). These observations imply that the rapid current decline in the nrx mutant synapses during tetanus is not due to the desensitization of postsynaptic glutamate receptors.

Next, we compared the rate of synaptic vesicle release in wild-type and nrx mutant synapses. To determine the quantal content of the nerve-evoked synaptic currents at the steady state, we measured the evoked currents and the spontaneous miniature currents at the steady state. The mean of evoked current was 72.0 ± 11.6 (n = 10) in the wild-type synapses and 40.5 ± 9.9 (n = 8) in the nrx mutant synapses (Fig. 2F). The averaged miniature current was 1.45 ± 0.42 (n = 824) in the wild-type synapses and 1.54 ± 0.40 (n = 960) in the nrx mutant synapses (Fig. 2F). Thus, the steady-state quantal content was 49.8 ± 8.0 quanta (n = 10) in the wild-type synapses compared with 26.2 ± 9.9 quanta (n = 8) in the nrx mutant synapses (Fig. 2F). This result indicates that synaptic vesicle release is impaired in the nrx mutant synapses during tetanic stimulation.

Intracellular Region of NRX Regulates Synaptic Vesicle Release—NRX is a cell adhesion molecule that contains a single transmembrane domain. Given that the synaptic vesicle release machinery operates in the cytosolic region of the presynaptic terminals (38), we questioned whether the intracellular region of NRX is required for synaptic vesicle release during tetanic stimulation. To test this possibility, we generated a C-termmally truncated NRX transgene p(LIAs-NRXΔC) in which the intracellular region of NRX was deleted and expressed this transgene in motor neurons. Previous studies have shown that the polarized targeting of NRX to synapses is regulated by their C-terminal sequences (39, 40). To examine whether NRXΔC is able to distribute into the presynaptic terminals of NMJs, we performed the antibody staining analysis. The results showed that full-length NRX was successfully distributed into the presynaptic terminals (Fig. 3, A and B). Consistent with previous findings, lack of the intracellular region of NRX led to massive NRXΔC retained in the motor neuron axons (Fig. 3, A and B). However, we found that a portion of NRXΔC was successfully distributed into the presynaptic terminals (Fig. 3, A and B). Meanwhile, the amount of NRXΔC distributed into the presynaptic terminals was comparable with that of full-length NRX (Fig. 3, A and B).

Because the truncated NRX was successfully distributed into the presynaptic terminals, we therefore performed the rescue experiments by using these transgenes. The synaptic current recording at the NMJ with 1.8 mM Ca2+ showed that the expression of NRXΔC failed to restore the defective short term synaptic depression in the nrx mutant synapses (Fig. 3, C–E), which indicates that the intracellular region of NRX is essential for synaptic vesicle release during tetanic stimulation. The PDZ-binding motif in the NRX molecule is able to associate with several molecules involved in the synaptic vesicle exocytosis machinery, including synaptotagmin and the PDZ domain-containing proteins CASK and Mints (16–18). To investigate the potential role of the PDZ-binding motif in the regulation of short term synaptic depression, we generated a NRX transgene with a deleted PDZ-binding motif (NRXΔ44) and performed the rescue experiments. Interestingly, the expression of NRXΔ44 failed to restore the rapid current decline in the first several dozen stimulations but recovered the reduced mean EJCs and quantal content at the steady state (Fig. 3, C–G). These observations strongly suggest that other intracellular regions of NRX also regulate synaptic vesicle release.

To examine the role of the cytoplasmic tail in synaptic vesicle release under normal conditions, we also recorded these rescue synapses under 0.5 mM Ca2+ conditions. Interestingly, expression of all NRX transgenes successfully restored the initial current amplitudes (Fig. 4, A–C), indicating the extracellular region of NRX is sufficient for coupling Ca2+ channels. These observations are supported by the previous study, which shows that extracellular domains of NRX participate in regulating synaptic transmission (41). Consistent with the recordings performed in 1.8 mM Ca2+ conditions, expression of full-length NRX and NRXΔC, but not NRXΔC, restored the reduced mean EJCs at the steady state (Fig. 4D). These results indicate that other intracellular regions of NRX regulate synaptic vesicle release at normal conditions.

NRX Associates with NSF in Vivo—To determine the molecular mechanism by which NRX regulates synaptic vesicle release during tetanic stimulation, we performed a yeast two-hybrid screen of a Drosophila cDNA library using the intracellular region of the Drosophila homolog of α-NRX as the bait. We identified several previously unreported binding proteins (Table 1), including NSF that mediates SNARE complex disassembly and plays an important role in synaptic vesicle release (5A) (42, 43). To evaluate the NRX/NSF interaction in vivo, we conducted a subcellular distribution analysis and co-immunoprecipitation assays. We separated the plasma membrane and synaptic vesicle membrane by sedimentation in a glycerol gradient (32). Quantitative Western blotting revealed that NRX existed predominantly in the plasma membrane-containing fractions where the plasma membrane marker Na+/K+-ATPase-α was present (Fig. 5B). Meanwhile, although NSF was present in both the plasma membrane-containing and non-plasma membrane-containing fractions, more than 50% of the NSF was in the plasma membrane-containing fractions (Fig. 5B). The co-immunoprecipitation experiment further demonstrated that NRX interacted with NSF in vivo (Fig. 4C). These results demonstrate that NRX associates with NSF at the presynaptic terminals.

In addition, we also measured the NSF-binding capacity of NRX with a pulldown assay. The results from this assay revealed that NRX bound to NSF in a concentration-dependent and saturable manner (Kd = 4.4 μM) (Fig. 5D). Furthermore, we examined the NSF-binding capacity of NRX in the fly head extracts with different Ca2+ concentrations. Interestingly, NRX showed the highest binding capacity with NSF in 0.5 mM Ca2+ condition and exhibited the declined binding ability with the increasing of Ca2+ concentration (Fig. 5E). These data indicate that the NSF-binding capacity of NRX is modulated by Ca2+ concentration.
NRX Binds to the D2 Domain of NSF—The intracellular region of NRX contains 122 amino acids, including a functional PDZ-binding motif (Fig. 6A) (3). We investigated which part within the intracellular region that mediates the interaction with NSF. The pulldown assay revealed that the purified NSF protein was able to bind with the fragments NRX(1730–1837), NRX(1730–1813), and NRX(1730–1833) but not with the fragments NRX(1730–1759) and NRX(1730–1787) (Fig. 6A). These observations indicate that amino acids 1788–1813 in the intracellular region of NRX mediate the interaction with NSF.

To further validate the NSF-binding sites in NRX, we expressed and purified the mutant NRX(H9004Δ988–1813) that deleted amino acids 1788–1813 of NRX. The pulldown assay revealed that NSF protein failed to bind with NRX(H9004Δ988–1813) (Fig. 6B).

An alignment analysis revealed that several amino acids are conserved in this region across different species (Fig. 6C), which suggests that the NRX/NSF interaction may occur in other species.

NSF has three distinct domains as follows: two homologous D domains (D1 and D2) and an N domain (Fig. 6D) (38). To determine which domain within the NSF protein interacts with NRX, pulldown assays using several constructs encoding truncated NSF fragments fused to GST were performed (Fig. 6D). The results revealed that purified NRX is able to bind to the full-length NSF protein (NSF(1–745)) as well as the truncated fragment NSF(1–588) but not the truncated fragments NSF(1–
Neurexin Regulates Synaptic Depression

![Graph](image)

**Figure 4. Intracellular region of NRX regulates synaptic vesicle release at 0.5 mM Ca²⁺ condition.** A, 40 Hz stimulation for 25 s with 0.5 mM Ca²⁺ in wild-type (black, n = 8), nrx mutant (gray, n = 10), and three indicated rescue (orange, n = 8; blue, n = 7; green, n = 8) synapses. 0.5 s of evoked peak EJC amplitudes are averaged at each time point and plotted as a function of time. B–D, quantification of the initial EJC amplitudes, the time for EJC amplitudes declining to 75%, and the steady-state EJC amplitudes in each genotype synapses.

**TABLE 1**

| Clone | Genes | Sequences | Molecular functions |
|-------|-------|-----------|--------------------|
| 54 tpmc4 | 1–152 | Calcium ion binding |
| 73 fhps9 | 157–180 | Peptidylprolyl cis-trans isomerase activity |
| 88 CG3978-PC | 2,065–2,289 | Calcium ion binding |
| X5 CG3978-PC | 2,107–2,289 | Same as above |
| X4 CG3978-PC | 2,090–2,289 | Same as above |
| 92 CG238 | 176–331 | Protein phosphatase 1 binding |
| 146 nrx3 | 37–247 | Sodium-potassium-exchanging ATPase activity |
| 174 tblh | 1–244 | Unknown |
| Y2 tblh | 1–244 | Unknown |
| 176 scb-11-PC | 1–188 | Transcription co-activator activity |
| 184 Cki α-3-PA | 1–280 | Protein kinase activity |
| G8 Cki α-3-PA | 1–181 | Same as above |
| E10 ephrin | 605–652 | Ephrin receptor binding |
| F4 CG1760 | 518–833 | Unknown |
| G5 rfabg | 364–493 | Lipid transporter activity; retinol binding |
| K1 rfabg | 464–623 | Same as above |
| L3 CG15576 | 1–123 | Unknown |
| M6 CG12374 | 335–422 | Metallocarboxypeptidase activity |
| X9 CG33123 | 1,102–1,182 | Aminocycl-tRNA editing activity |
| X7 ntv | 482–715 | Chromatin binding |
| YH1 comt | 351–745 | ATPase activity |
| Y4 med26 | 1,216–1,546 | Transcription factor activity |
| YL7 CG7102 | 364–621 | Unknown |

513), NSF(1–389), or NSF(1–175) (Fig. 6D). These results suggest that the amino acid sequence 514–588 in the NSF protein is a critical component of the NRX interaction with NSF. An alignment analysis revealed that this region of NSF (Fig. 6E), which is partially within the D2 domain essential for the hexamerization of NSF (44, 45), is highly conserved across species.

**NRX/NSF Interaction Is Essential for NSF Recruitment**—To determine the potential role of the NRX/NSF interaction in the regulation of NSF function and subsequent synaptic vesicle release, we examined the distribution of NSF in nrx mutant synapses. The distribution of NSF was determined relative to the markers for the postsynaptic membrane as well as active zone regions of synapse where synaptic vesicles dock and fuse. In the wild-type NMJs, the NSF occupied the cytosolic regions within the presynaptic boutons and existed predominantly near the presynaptic membrane in a similar ratio to the postsynaptic marker DLG (Fig. 7, A–C). In contrast, in the nrx mutant NMJs, a large portion of the NSF fell into the cytosolic region within the boutons (Fig. 7, A–C). To validate the distribution of NSF within the wild-type and nrx mutant synapses, the plasma and synaptic vesicle membranes were separated by sedimentation in a glycerol gradient. Quantitative Western blotting revealed that the sedimentation profile of NSF was shifted significantly from the plasma membrane fractions to the cytosolic fractions in the nrx mutant synapses compared with the wild-type synapses (Fig. 7D). These findings indicate that the loss of NRX results in an altered NSF distribution at the presynaptic terminals.

To address whether the NRX/NSF interaction facilitates the recruitment of NSF to the presynaptic terminals, we performed rescue experiments. Interestingly, the expression of the full-length NRX transgene p(USAS-NRX) but not the C-terminally truncated Neurexin transgene p(USAS-NRXΔC) rescued the abnormal NSF distribution in the nrx mutant synapses (Fig. 7, E and F). These observations strongly suggest that the NRX/NSF interaction is essential for the recruitment of NSF to the presynaptic terminals.

NSF is known to mediate SNARE complex disassembly (42, 43). A biochemical analysis has shown that SDS-resistant 7S SNARE complexes accumulate in comt mutant flies following the disruption of NSF activity (43). We therefore asked whether the loss of the NRX/NSF interaction would affect SNARE complex disassembly. Western blotting analysis revealed that SDS-resistant 7S SNARE complex was accumulated in nrx mutants compared with wild-type flies (Fig. 7, G and H). These data suggest that the NRX/NSF interaction promotes SNARE complex disassembly and the subsequent synaptic vesicle release during tetanic stimulation.

Taken together, these data support a model in which NRX/NSF interaction facilitates the recruitment of NSF in the presynaptic terminals and restricts the movement of NSF at rest conditions. With the increase of terminal Ca²⁺ concentration during stimulation, NRX releases NSF to facilitate the disassembly of SNARE complexes.

**Discussion**

**NRX Facilitates Repetitive Synaptic Vesicle Release**—Previous studies have shown that the α-NRXs functionally couple Ca²⁺ channels to the presynaptic machinery to mediate synaptic vesicle exocytosis (4, 6) and that defective synaptic vesicle release can be restored with 1 mM Ca²⁺ (4). In our experiments, 1.8 mM Ca²⁺ was used to eliminate the effects of abnormal Ca²⁺ sensitivity in neurotransmitter release. Under this condition, we showed that nrx mutant synapses exhibit rapid short term synaptic depression and reduced quantal content of nerve-evoked synaptic currents at the steady state during tetanic stimulation. These observations suggest that NRX regulates activity-dependent synaptic plasticity. Similar observations have been reported in the fast-twitch diaphragm muscle of the...
α-NRX double knock-out mouse, which indicates that presynaptic efficacy but not presynaptic homeostatic plasticity is normal under basal conditions (46).

Deficits in both the presynaptic neurotransmitter release machinery and the postsynaptic neurotransmitter receptors may cause rapid short term synaptic depression (31, 37). As a presynaptic adhesion molecule, NRX interacts with the postsynaptic adhesion molecule, Neurexin, and bridges the synaptic cleft that aligns the presynaptic neurotransmitter release machinery with the postsynaptic neurotransmitter receptors. It has been suggested that the activity-dependent regulation of the NRX/Neurilgin interaction mediates learning-related synaptic remodeling and long term facilitation (47). A recent study reveals that the alternative splicing of presynaptic NRX-3 controls postsynaptic AMPA receptor trafficking and long term plasticity in mice (48). In this study, we showed that the kinetics of the individual synaptic currents were stable during tetanic stimulation in the nrx mutant synapses. Moreover, we showed that NRX mediated synaptic plasticity through the presynaptic machinery, which was supported by our rescue experiments.

NRX Interacts with NSF—The intracellular sequence of NRX, including the C-terminal PDZ-binding motif, is identical across several vertebrate and invertebrate species. Mammalian NRXs only possesses a short cytoplasmic tail (55 amino acids), including a PDZ-binding motif (36). To identify the potential binding partners, several laboratories have conducted yeast two-hybrid screening by using the cytosolic tail of mammalian NRX as a bait. Two PDZ-containing proteins, CASK and syntenin, have been identified in the previous screening (17, 19), and further studies show that the binding between CASK and NRX is abolished by deletion of the last three amino acids of the intracellular C-terminal region of NRX (17). However, it seems that the previous screenings are not saturated, as the NRX-interacted protein Mints was not identified in these screenings (18).

Drosophila NRX contains a long cytoplasmic tail (122 amino acids), including a functional PDZ-binding motif (3). Thus, it is possible that the long cytoplasmic tail may associate with some partners in a PDZ-independent manner. In this study, normalized Drosophila cDNA libraries were used in our screening, which helps to identify the binding partner with low abundance. In our screening, 23 cDNAs clones that encode the fragments of 18 proteins were recovered. Our previous study has demonstrated that the cytoplasmic tail of NRX associates with Rfabg and facilitates the retinol transport (29). In this study, we identified the intracellular region of NRX binds with NSF through the amino acid sequence 1788–1813 but not the PDZ-binding motif of NRX. We found that this NRX/NSF interaction is essential for the NSF recruitment to the presynaptic terminals and plays an important role in synaptic vesicle release. In addition, we provided multiple lines of evidence that NRX associates with NSF at the presynaptic terminals. An alignment analysis of the NSF-binding site of NRX revealed that this sequence is conserved across different species. Moreover, this sequence is present across many proteins that serve different functions, which implies that this sequence may be essential for protein interactions.
Each NSF molecule contains an N-terminal domain that is responsible for the interaction with α-SNAP and the SNARE complex, a low affinity ATP-binding domain (D1 domain) whose hydrolytic activity is associated with NSF-driven SNARE complex disassembly and a C terminal high affinity ATP-binding domain (D2 domain) (44, 45, 50, 51). In this study, we mapped the NRX-binding sites of NSF to the D2 domain, which mediates the ATP-dependent oligomerization of NSF (45, 52). An alignment analysis showed that both the D2 domain in NSF and the NSF-binding site in NRX are highly conserved. This result suggests that the NRX/NSF interaction may occur in other species, a possibility that needs to be investigated further.

It has been documented that the D2 domain of NSF is essential for its hexamerization (44, 45). In this study, we show that NRX exhibits a declined NSF binding capacity in high Ca\(^{2+}\) concentration. These observations suggest that NSF can be released from NRX under stimulation. Although NRX and NSF do not bind with Ca\(^{2+}\) directly, it is possible that they undergo Ca\(^{2+}\)-signaling-dependent modifications or bind with some Ca\(^{2+}\)-binding proteins. In our yeast two-hybrid screening, one potential Ca\(^{2+}\)-binding protein (CG33978) has been identified. However, the mechanism of how NRX releases NSF under high Ca\(^{2+}\) concentration need to be further investigated.

Neurexin Regulates Synaptic Depression—Previous studies have shown that the PDZ-binding motif of NRX associates with several molecules involved in the synaptic vesicle exocytosis machinery, including synaptotagmin and the PDZ domain-containing proteins CASK and Mints (16–18). Synaptotagmin functions as a Ca\(^{2+}\) sensor and controls synaptic membrane fusion machinery (26). Adaptor protein Mints regulates presynaptic vesicle release (53), whereas the other adaptor protein CASK is not essential for the Ca\(^{2+}\)-triggered presynaptic release (54). In contrast, CASK interacts with NRX and protein 4.1 to form a trimeric complex and regulates synapse formation (55, 56). The process of synaptic vesicle release includes several consecutive steps, docking, priming, and fusion (26). Depending on the stimulation given to synapses, different synaptic vesicle trafficking steps become rate-limiting for synaptic vesicle release. This causes short term changes in synaptic transmission that determine many higher brain functions such as sound localization, sensory adaptation, or even working memory (57). Thus, the PDZ-binding motif-linked synaptic vesicle exocytotic machinery might regulate the different steps during synaptic vesicle release. Indeed, nrx mutant synapses that express NRX\(^{34}\) exhibit a rapid current decline over the first several...
dozen stimulations. In contrast, the expression of NRX<sup>54</sup> largely restores the reduced steady-state mean EJCs and quantal content in neurexin mutant synapses. Together with the C-terminally truncated NRX rescue experiments, these data suggest that intracellular regions of NRX other than the PDZ-binding motif also regulate short term synaptic depression. The existing literature extensively documents the roles of NSF in SNARE complex disassembly and short term synaptic depression (38, 43, 58). In this study, we extended these findings to show that the NRX/NSF interaction facilitates the recruitment of NSF to the presynaptic terminals and promotes the subsequent SNARE complex disassembly.

Previous studies have established that the NSF hexamer serves as the only active form for SNARE complex disassembly (23, 45, 49, 50, 59). In this study, our binding assay showed that purified NRX binds to NSF in a concentration-dependent and saturable manner. NRXs appear to serve as scaffold proteins to recruit NSF, and the NRX/NSF interaction may promote SNARE complex disassembly in vivo. Live image studies have shown that NSF mutant (i.e. comt) synapses exhibit defective NSF re-distribution during tetanic nerve stimulation (28). In this study, immunocytochemical and sedimentation analyses revealed that a lack of the NRX/NSF interaction results in an altered distribution of NSF and an accumulation of 7S SNARE complexes. Our results imply that NRX/NSF interaction serves as a potential mechanism to restrict the mobilization of NSF.

Our electrophysiological recordings revealed that synaptic depression was comparable between nrx mutant synapses and wild-type synapses in response to low frequency stimulations. The long intervals between low frequency stimulations may allow the remaining NSF to disassemble the SNARE complexes and generate enough free t-SNARE for the subsequent synaptic vesicle fusion events. Another possibility that needs to be investigated further is that tetanic stimulation may have other effects on the re-distribution of NSF.

In summary, our study provides evidence that the NRX/NSF interaction recruits NSF to the presynaptic terminals and promotes SNARE complex disassembly. Our findings have revealed a previously unknown role of NRX in the regulation of neurotransmitter release and provide a linkage between the presynaptic adhesion molecules and the presynaptic plasticity machinery.

Acknowledgments—We thank Dr. Manzoor A. Bhat for providing the nrx<sup>273</sup> flies, and Dr. Zhengping Jia, Dr. Wei Lu, Dr. Zikai Zhou, and members of the Han laboratory for their critical comments on the manuscript.

References
1. Scheiffele, P. (2003) Cell-cell signaling during synapse formation in the CNS. Annu. Rev. Neurosci. 26, 485–508
Neurexin Regulates Synaptic Depression

2. Dean, C., Scholl, F. G., Choij, J., DeMaria, S., Berger, J., Isacoff, E., and Scheiffele, P. (2003) Neurexin mediates the assembly of presynaptic terminals. Nat. Neurosci. 6, 708–716

3. Zeng, X., Sun, M., Liu, L., Chen, F., Wei, L., and Xie, W. (2007) Neurexin-1 is required for synapse formation and larval associative learning in Drosophila. FEBS Lett. 581, 2509–2516

4. Li, J., Ashley, J., Budnik, V., and Bhat, M. A. (2007) Crucial role of neurexin in proper active zone apposition to postsynaptic densities, synaptic growth, and synaptic transmission. Neuron 55, 741–755

5. Yamagata, M., Sanes, J. R., and Weiner, J. A. (2003) Synaptic adhesion molecules. Curr. Opin. Cell Biol. 15, 621–632

6. Missler, M., Zhang, W., Rohllmann, A., Kattenstroth, G., Hammer, R. E., Gottmann, K., and Südhof, T. C. (2003) α-Neurexins couple Ca2+ channels to synaptic vesicle exocytosis. Nature 423, 939–948

7. Ichihchenko, K., Hata, Y., Nguyen, T., Ullrich, B., Missler, M., Moomaw, C., and Südhof, T. C. (1995) Neurexin 1: a splice site-specific ligand for β-neurexins. Cell 81, 435–443

8. Boucard, A. A., Chubykin, A. A., Comolletti, D., Taylor, P., and Südhof, T. C. (2005) A splice code for trans-synaptic cell adhesion mediated by binding of neurelin 1 to α- and β-neurexins. Neuron 48, 229–236

9. Sugiya, S., Saito, F., Tang, J., Satz, J., Campbell, K., and Südhof, T. C. (2001) A stoichiometric complex of neurexins and dystroglycan in brain. J. Cell Biol. 154, 435–445

10. Missler, M., Hammer, R. E., and Südhof, T. C. (1998) Neurexophilin binding to α-neurexins. A single LNS domain functions as an independently folding ligand-binding unit. J. Biol. Chem. 273, 34716–34723

11. Missler, M., and Südhof, T. C. (1998) Neurexophilins form a conserved family of neuropeptide-like glycoproteins. J. Neurosci. 18, 3630–3638

12. Petrenko, A. G., Ullrich, B., Missler, M., Krasnoperov, V., Rosahl, T. W., and Südhof, T. C. (1996) Structure and evolution of neurexophilins. J. Neurosci. 16, 4360–4369

13. de Wit, J., Sylwestrak, E., O’Sullivan, M. L., Otto, S., Tiglio, K., Savas, J. N., Yates, J. R., 3rd, Comolletti, D., Taylor, P., and Ghosh, A. (2009) LRTM2 interacts with Neurexin1 and regulates excitatory synapse formation. Neuron 64, 799–806

14. Ko, J., Fuccillo, M. V., Malenka, R. C., and Südhof, T. C. (2009) LRTM2 functions as a neurexin ligand in promoting excitatory synapse formation. Neuron 64, 791–798

15. Uemura, T., Lee, S. J., Yasumura, M., Takeuchi, T., Yoshida, T., Ra, M., Taguchi, R., Sakimura, K., and Mishina, M. (2010) Trans-synaptic interaction of GluR2 and Neurexin through Cbln1 mediates synapse formation in the cerebellum. Cell 141, 1068–1079

16. Hata, Y., Davletov, B., Petrenko, A. G., Jahn, R., and Südhof, T. C. (1993) Interaction of synaptotagmin with the cytoplasmic domains of neurexins. Neuron 10, 307–315

17. Hata, Y., Butz, S., and Südhof, T. C. (1996) CASK: a novel dlg/PDS95 homolog with an N-terminal calmodulin-dependent protein kinase domain identified by interaction with neurexins. J. Neurosci. 16, 2488–2494

18. Biederer, T., and Südhof, T. C. (2000) Mints as adaptors. Direct binding to N- and P/Q-type Ca2+ channels is required for synapse development and function at the Drosophila neuromuscular junction. J. Neurosci. 31, 687–699

19. Littleton, I. T., Chapman, R. E., Kreber, R., Garment, M. B., Carlson, S. D., and Ganetzky, B. (1998) Temperature-sensitive paralytic mutations demonstrate that synaptic exocytosis requires SNARE complex assembly and disassembly. Neuron 21, 401–413

20. Rowen, L., Young, J., Birditt, B., Kaur, A., Madan, A., Philipps, D. L., Qin, S., Minx, P., Wilson, R. K., Hood, L., and Gravelle, B. R. (2002) Analysis of the human neurexin genes: alternative splicing and the generation of protein diversity. Genomics 79, 587–597

21. Littleton, I. T., Barnard, R. J., Slind, J., Chapman, R. E., and Sigrist, S. J. (2012) Cooperation of Syd-1 with Neurexin through Cbln1 mediates synapse formation. Nat. Rev. Mol. Cell Biol. 13, 20–26

22. Delgado, R., Maureira, C., Oliva, C., Kidokoro, Y., and Labarca, P. (2000) Size of vesicle pools, rates of mobilization, and recycling at neuromuscular synapses of a Drosophila mutant, shibire. Neuron 28, 941–953

23. Jahn, R., and Scheller, R. H. (2006) SNAREs—engines for membrane fusion. Nat. Rev. 7, 631–643

24. Fairless, R., Masius, H., Rohllmann, A., Heupel, K., Ahmad, M., Reissner, C., Dresbach, T., and Missler, M. (2008) Polarized targeting of neurexins to synapses is regulated by their C terminal sequences. J. Neurosci. 28, 12969–12981

25. Oswald, D., Khorrarmashahi, O., Gupta, V. K., Banovic, D., Depner, H., Fouquet, W., Wichmann, C., Mertel, S., Eimer, S., Reynolds, E., Holt, M., Aberle, H., and Sigrist, S. J. (2012) Cooperation of Syd-1 with Neurexin synchronizes pre- with postsynaptic assembly. Nat. Neurosci. 15, 1219–1226

26. Zhang, W., Rohllmann, A., Sargsyan, V., Aramuni, G., Hammer, R. E., Südhof, T. C., and Missler, M. (2005) Extracellular domains of α-neurexins participate in regulating synaptic transmission by selectively affecting N- and P/Q-type Ca2+ channels. J. Neurosci. 25, 4330–4342

27. Littleton, I. T., Barnard, R. J., Slind, J., Chapman, R. E., and Ganetzky, B. (2001) SNARE-complex disassembly by NSF follows synaptic-vesicle fusion. Proc. Natl. Acad. Sci. U.S.A. 98, 12233–12238

28. Tolar, L. A., and Pallack, L. (1998) NSF function in neurotransmitter release involves rearrangement of the SNARE complex downstream of synaptic vesicle docking. J. Neurosci. 18, 10250–10256

29. Lenzen, C. U., Thomsen, J., Bruce, J., Weis, W. S., and Weis, W. I. (1998) Crystal structure of the hexamerization domain of N-ethylmaleimide-sensitive fusion protein. Cell 94, 525–536

30. Yu, R. C., Hanson, P. I., Jahn, R., and Brünger, A. T. (1998) Structure of the ATP-dependent oligomerization domain of N-ethylmaleimide sensitive factor complexed with ATP. Nat. Struct. Biol. 5, 803–811
46. Sons, M. S., Busche, N., Strenzke, N., Moser, T., Ernsberger, U., Mooren, F. C., Zhang, W., Ahmad, M., Steffens, H., Schomburg, E. D., Plomp, J. J., and Missler, M. (2006) α-Neurexins are required for efficient transmitter release and synaptic homeostasis at the mouse neuromuscular junction. *Neuroscience* **138**, 433–446

47. Choi, Y. B., Li, H. L., Kassabov, S. R., Jin, I., Puthanveettil, S. V., Karl, K. A., Lu, Y., Kim, J. H., Bailey, C. H., and Kandel, E. R. (2011) Neurexin-neuroligin transsynaptic interaction mediates learning-related synaptic remodeling and long term facilitation in aplysia. *Neuron* **70**, 468–481

48. Aoto, J., Martinelli, D. C., Malenka, R. C., Tabuchi, K., and Südhof, T. C. (2013) Presynaptic neurexin-3 alternative splicing trans-synaptically controls postsynaptic AMPA receptor trafficking. *Cell* **154**, 75–88

49. Hanson, P. I., Roth, R., Morisaki, H., Jahn, R., and Heuser, J. E. (1997) Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell* **90**, 523–535

50. Whiteheart, S. W., Rossnagel, K., Buhrow, S. A., Brunner, M., Jaenicke, R., and Rothman, J. E. (1994) N-ethylmaleimide-sensitive fusion protein: a trimeric ATPase whose hydrolysis of ATP is required for membrane fusion. *J. Cell Biol.* **126**, 945–954

51. Fleming, K. G., Hohl, T. M., Yu, R. C., Müller, S. A., Wolpensinger, B., Engel, A., Engelhardt, H., Brünger, A. T., Sollner, T. H., and Hanson, P. I. (1998) A revised model for the oligomeric state of the N-ethylmaleimide-sensitive fusion protein, NSF. *J. Biol. Chem.* **273**, 15675–15681

52. Tagaya, M., Wilson, D. W., Brunner, M., Arango, N., and Rothman, J. E. (1993) Domain structure of an N-ethylmaleimide-sensitive fusion protein involved in vesicular transport. *J. Biol. Chem.* **268**, 2662–2666

53. Ho, A., Morishita, W., Atasoy, D., Liu, X., Tabuchi, K., Hammer, R. E., Malenka, R. C., and Südhof, T. C. (2006) Genetic analysis of Mint/X11 proteins: essential presynaptic functions of a neuronal adaptor protein family. *J. Neurosci.* **26**, 13089–13101

54. Atasoy, D., Schoch, S., Ho, A., Nadasy, K. A., Liu, X., Zhang, W., Mukherjee, K., Nosyreva, E. D., Fernandez-Chacon, R., Missler, M., Kavalali, E. T., and Südhof, T. C. (2007) Deletion of CASK in mice is lethal and impairs synaptic function. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 2525–2530

55. Gokce, O., and Südhof, T. C. (2013) Membrane-tethered monomeric neurexin LNS-domain triggers synapse formation. *J. Neurosci.* **33**, 14617–14628

56. Biederer, T., and Sudhof, T. C. (2001) CASK and protein 4.1 support F-actin nucleation on neurexins. *J. Biol. Chem.* **276**, 47869–47876

57. Wojcik, S. M., and Brose, N. (2007) Regulation of membrane fusion in synaptic excitation-secretion coupling: speed and accuracy matter. *Neuron* **55**, 11–24

58. Kawasaki, F., and Ordway, R. W. (2009) Molecular mechanisms determining conserved properties of short term synaptic depression revealed in NSF and SNAP-25 conditional mutants. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 14658–14663

59. Nagiec, E. E., Bernstein, A., and Whiteheart, S. W. (1995) Each domain of the N-ethylmaleimide-sensitive fusion protein contributes to its transport activity. *J. Biol. Chem.* **270**, 29182–29188