Regulation of Neurotransmitter Release Kinetics by NSF

Felix E. Schweizer, Thomas Dresbach, William M. DeBello, Vincent O'Connor, George J. Augustine,* Heinrich Betz

NSF (*N*-ethylmaleimide–sensitive factor) is an adenosine triphosphatase (ATPase) that contributes to a protein complex essential for membrane fusion. The synaptic function of this protein was investigated by injecting, into the giant presynaptic terminal of squid, peptides that inhibit the ATPase activity of NSF stimulated by the soluble NSF attachment protein (SNAP). These peptides reduced the amount and slowed the kinetics of neurotransmitter release as a result of actions that required vesicle turnover and occurred at a step subsequent to vesicle docking. These results define NSF as an essential participant in synaptic vesicle exocytosis that regulates the kinetics of neurotransmitter release and, thereby, the integrative properties of synapses.

Information transfer between neurons depends on the amount and duration of synaptic transmitter release. Although many processes are known to regulate the amount of neurotransmitter released (*1*), the molecular mechanisms responsible for determining the time course of transmitter release are unknown (*2*). For example, none of the various proteins implicated in neurotransmitter release (*3*) has been assigned a role in regulating release kinetics—in part, because most analyses of these proteins have been performed under steady-state conditions in vitro. We have now studied an intact synapse in order to evaluate the physiological role of the cytosolic ATPase NSF in neurotransmitter release.

NSF proteins are composed of three domains: a variable NH_2-terminus, a central D1 domain, and a COOH-terminal D2 trimerization domain (*4*). We concentrated on the D1 region, which is essential for many of the actions of NSF (*5*). Peptide fragments corresponding to this region were injected into the giant presynaptic terminal of squid (*6*) and their effects on transmitter release were monitored. Sequencing of a cDNA encoding most of squid NSF (*7*) indicated that the D1 region of the squid protein is ~75% identical to that of NSF proteins from mammals and *Drosophila*, and 67% identical to that of the yeast homolog Sec18 (Fig. 1A). Predictions of hydrophilicity, higher order structure, and evolutionary conservation of sequence motifs in squid NSF were used to identify potential sites of protein-protein interaction, on the basis of which six peptides (Fig. 1B) were synthesized and tested for their ability to interfere with NSF function.

Two of the peptides, NSF-2 and NSF-3, markedly (>50%) inhibited neurotransmitter release when injected into the squid giant presynaptic terminal (Fig. 2, A, B, and D) (*8*, *9*). NSF-1 inhibited release to a lesser extent (33 ± 7%; mean ± SEM, n = 10), whereas NSF-4, NSF-5, or NSF-6 reduced synaptic transmission by <5% when injected at concentrations similar to or higher than those of the active peptides. The inhibitory effects of NSF-2 and NSF-3 were apparent within minutes of the onset of the injection, reached a maximum soon after injection ceased, and were completely reversible (Fig. 2, B and D). Because recovery paralleled the loss of coinjected fluorescent dextran (Fig. 2D), it was probably the result of diffusion of the peptide out of the synaptic terminal (*8*).

A peptide (scNSF-2) with an amino acid composition identical to that of NSF-2, but with a scrambled sequence, had no effect on transmitter release (Fig. 2C). Moreover, replacing the central glycine of NSF-3 by glutamate yielded a peptide (comNSF-3) that was inactive even at concentrations higher than those of NSF-3 required to inhibit synaptic transmission completely (Fig. 2D). These two results indicate that NSF-2 and NSF-3 act in a sequence-specific manner, and, thus, that their effects reflect an action related to NSF. A point mutation in the *comatose* gene causes a change in the sequence of *Drosophila* NSF identical to that in comNSF-3 (*10*) and leads to impairment of synaptic transmission and paralysis (*11*). Our data indicate that this paralysis likely results from destabilization of a protein-protein interaction required for transmitter release, rather than from inhibition of the many other processes that contribute to synaptic transmission (*11*).

SNAP (soluble NSF attachment pro-

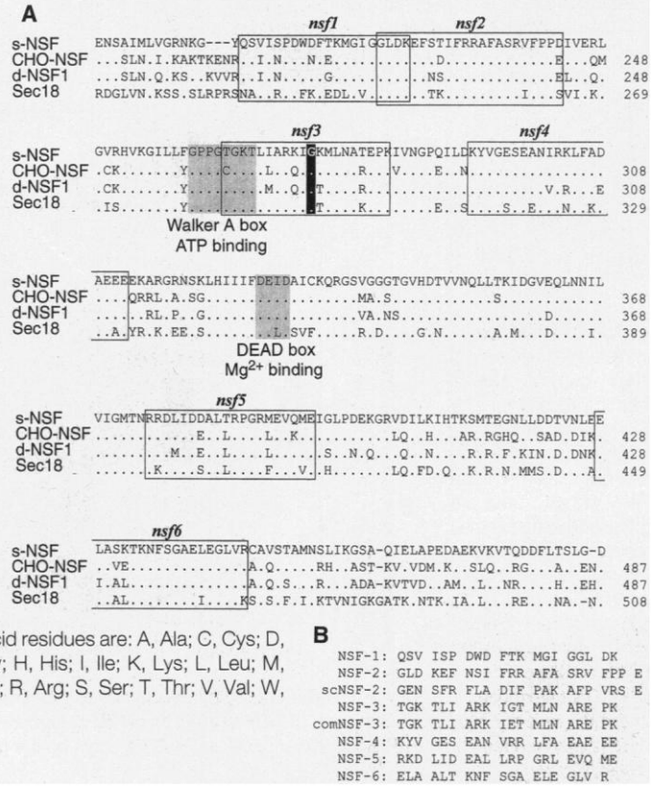

Fig. 1. (**A**) Deduced primary structures of the D1 domain of NSF from squid (s-NSF), mammals (CHO-NSF), *Drosophila* (d-NSF1), and *Saccharomyces cerevisiae* (Sec18). Dots indicate residues identical to those of s-NSF. The glycine highlighted by the black bar is substituted by a glutamic acid residue in the *Drosophila comatose* mutant. Open boxes indicate the positions (*nsf1* to *nsf6*) corresponding to peptides used for microinjection; shaded areas indicate binding sites for ATP and Mg^{2+}. Residue numbers are indicated on the right. (**B**) Amino acid sequences of peptides used in this study. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

F. E. Schweizer, W. M. DeBello, G. J. Augustine, Department of Neurobiology, Duke University Medical Center, Durham, NC 27710, USA, and Marine Biological Laboratory, Woods Hole, MA 02543, USA.
T. Dresbach, V. O'Connor, H. Betz, Department of Neurochemistry, Max Planck Institute for Brain Research, 60528 Frankfurt, Germany.

*To whom correspondence should be addressed. E-mail: georgea@neuro.duke.edu

tein) is required for the interaction of NSF with its membrane receptors, known as SNAREs (SNAP receptors) (*12*), and regulates the ATPase activity of NSF (*13*). Both NSF-2 and NSF-3 inhibited SNAP-stimulated ATP hydrolysis by NSF in vitro (*14*), whereas the mutant comNSF-3 peptide was inactive (Fig. 3A). The peptides inhibited SNAP-stimulated ATPase activity at concentrations (Fig. 3B) similar to those that inhibit transmitter release (Fig. 2D). We also tested the responsiveness of postsynaptic receptors by photolysis of caged glutamate (*15*). Presynaptic injection of NSF-2 had no effect on responses to photoreleased glutamate (Fig. 3C), showing that postsynaptic glutamate receptors were unaffected by presynaptically injected NSF peptides. These results indicated that NSF and its regulation by SNAP are essential for neurotransmitter release.

In addition to reducing the initial slope of excitatory postsynaptic potentials (EPSPs), NSF-2 and NSF-3 also appeared to prolong these responses, suggesting that they reduce not only the magnitude but also slow the kinetics of transmitter release. We tested this possibility by voltage-clamping the postsynaptic axon to avoid the effects of voltage-dependent conductances that obscure the EPSP time course. The resultant measurements of excitatory postsynaptic currents (EPSCs) revealed that NSF-2 and NSF-3 reduced the amount of transmitter release, as indicated by a decrease in both the amplitude and the integral of the EPSCs. In addition, both peptides increased the synaptic delay and markedly slowed both the onset and the decay of EPSCs (Fig. 4, A and B, Fig. 5A, and Table 1) (*16*). Blockers of glutamate uptake affected neither the time course nor the amplitude of transmitter release (n = 3), making it unlikely that the peptides acted by altering the glutamate transient in the synaptic cleft (*17*). Such slowing of the EPSC is highly unusual and was not simply a consequence of reduced transmitter release. For example, slowing was not observed with the other NSF peptides nor with any other manipulation that reduced EPSC amplitude (*18–20*), including injection of the synaptotagmin peptide Pep20 (*8*) (Fig. 4C), reducing the extracellular Ca^{2+} concentration (Fig. 4D), or evoked synaptic depression by a high-frequency train of presynaptic action potentials (Fig. 5). On the basis of these results, as well as our observations that NSF peptides had no effect on postsynaptic receptors (Fig. 3C) and that they prolonged both the onset and the decay of the EPSC (Fig. 5), we conclude that NSF-2 and NSF-3 alter EPSC kinetics by changing the time course of transmitter release (*2, 21*).

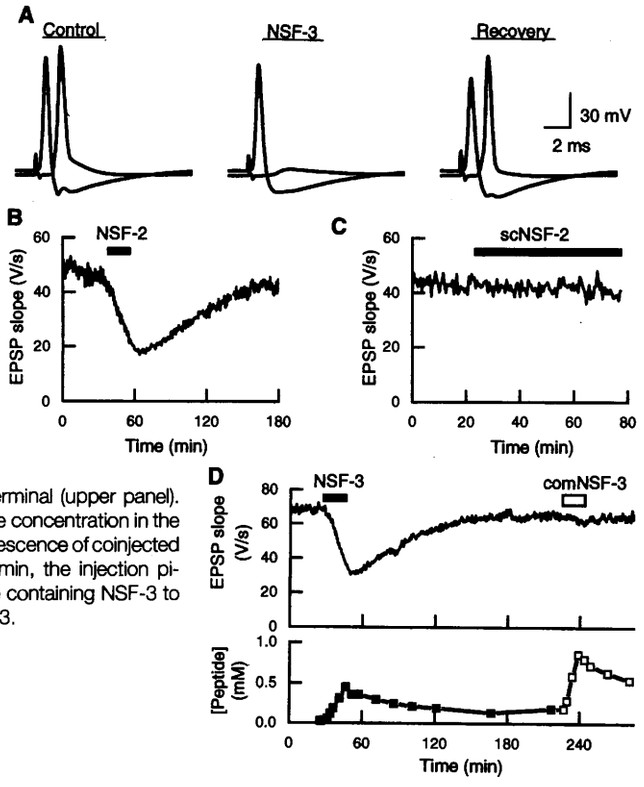

Fig. 2. Inhibition of neurotransmitter release by NSF peptides. (**A**) Examples of pre- and postsynaptic potentials recorded before, during, and 5 hours after NSF-3 injection (left, middle, and right panels, respectively). (**B** and **C**) Inhibition of transmitter release by NSF-2 (B) but not by scrambled NSF-2 (scNSF-2) (C). Bars indicate duration of peptide microinjection. (**D**) Differential effects of injections of NSF-3 (solid bar) and comNSF-3 (open bar) into the same presynaptic terminal (upper panel). Lower panel indicates peptide concentration in the terminal as estimated by fluorescence of coinjected fluorescein-dextran. At 180 min, the injection pipette was changed from one containing NSF-3 to another containing comNSF-3.

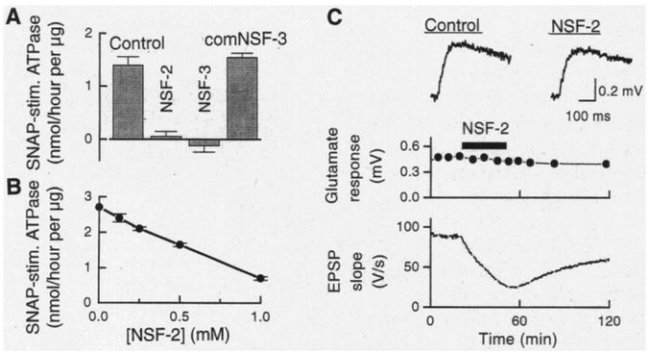

Fig. 3. Inhibition of SNAP-stimulated ATPase activity of NSF, but not of postsynaptic receptors, by NSF peptides. (**A**) Effect of peptides (1 mM) on SNAP-stimulated ATPase activity of NSF. Data are expressed as nanomoles of phosphate per hour per microgram of NSF and are means ± SEM (n = 3). (**B**) Dose-dependent inhibition of SNAP-stimulated ATPase activity of NSF by NSF-2. Data are means ± SEM (n = 3). (**C**) Postsynaptic responses to flash photolysis of caged glutamate immediately before (control) and at the end of (NSF-2) injection of NSF-2 (upper panel). Presynaptic injection of NSF-2 (bar) reduced the EPSP slope (lower panel) but had no effect on the glutamate response (middle panel).

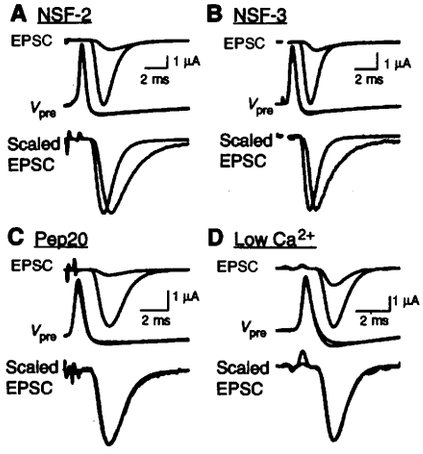

Fig. 4. Effect of NSF peptides on the kinetics of transmitter release. Presynaptic potentials (V_{pre}), postsynaptic currents (EPSCs), and the same EPSCs scaled to identical peak amplitudes (scaled) are shown during injection of NSF-2 (**A**), NSF-3 (**B**), or synaptotagmin-derived Pep20 (**C**) or the lowering of the extracellular Ca^{2+} concentration (**D**). Stimulus artifact transients were blanked out and the smallest, scaled traces were additionally filtered.

NSF peptides might have influenced release kinetics by altering Ca^{2+} entry into the terminal through presynaptic Ca^{2+} channels (*22*). However, voltage-clamp measurements of presynaptic Ca^{2+} currents indicated that this was not the case (Fig. 6A). On average, NSF-2 had no effect on the amplitude or the time course of the presynaptic Ca^{2+} current (3.5 ± 6.4% enhancement of current measured at 6 ms, $n = 7$) while inhibiting (by 66 ± 5%) and slowing (by 31 ± 5%) the postsynaptic current.

There are two plausible explanations for the ability of NSF peptides to influence release kinetics on the millisecond time scale without altering Ca^{2+} entry: These peptides could act either by directly interfering with the fusion of docked vesicles (*12*) or by slowing the fusion of vesicles indirectly by affecting a step that precedes (*23*, *24*) or follows (*25*) fusion. Whereas the latter hypothesis predicts that NSF peptides will block transmitter release only after vesicle turnover depletes available vesicles, the former predicts that inhibition will be independent of vesicle turnover. We found that NSF-2 blocked synaptic transmission only when the synapse was active (Fig. 6B) (*26*). Thus, NSF appears to act at a step other than fusion, yet it regulates both the amount and the timing of neurotransmitter release.

Recent in vitro studies of yeast vacuolar fusion suggest that NSF acts before vesicles dock (*24*). If the NSF peptides inhibit release by preventing docking, they should reduce the number of docked vesicles and induce the accumulation of upstream, nondocked vesicles. However, electron microscopy revealed the opposite effect. Terminals injected with NSF-2 (Fig. 6D) exhibited an 18% increase in the number of docked vesicles (those located within 50 nm of the presynaptic plasma membrane) compared with control terminals (Fig. 6C). Thus, the peptides affected a step that follows vesicle docking but precedes vesicle fusion. The total number of vesicles was reduced by 46% in the terminals injected with NSF-2 (Fig. 6E), presumably because of a contribution of NSF-dependent reactions to the endocytotic branch of the vesicle cycle (*19*). This decrease in the number of vesicles is not likely responsible for the changes in release because the number of docked vesicles was increased, potentially providing more vesicles for rapid fusion. Furthermore, other treatments that reduce the number of synaptic vesicles do not change release kinetics (*19*, *20*). We suggest that the kinetic effect of the peptides is related to the role of NSF in exocytosis, even though this protein apparently acts during

Table 1. Effects of experimental treatments on EPSC properties. EPSCs were measured before and at the peak of injection of the indicated peptides, or before and at the end of a presynaptic train of stimuli (1 Hz, 30 s). A slowing of 100% is equivalent to a doubling of the EPSC onset or decay time constants relative to control. Data are means ± SEM.

Treatment	No. of injections (reversals) or trains	Amplitude inhibition (%)	Onset slowing (%)	Decay slowing (%)
NSF-1	10 (5)	33 ± 7	3 ± 2	9 ± 5
NSF-2	56 (29)	72 ± 3	48 ± 5	110 ± 14
NSF-3	6 (5)	73 ± 8	54 ± 27	97 ± 36
comNSF-3	4	20 ± 9	3 ± 8	8 ± 5
Pep20	3 (2)	69 ± 13	6 ± 6	24 ± 10
Stimulus train	8	64 ± 4	1 ± 3	8 ± 3

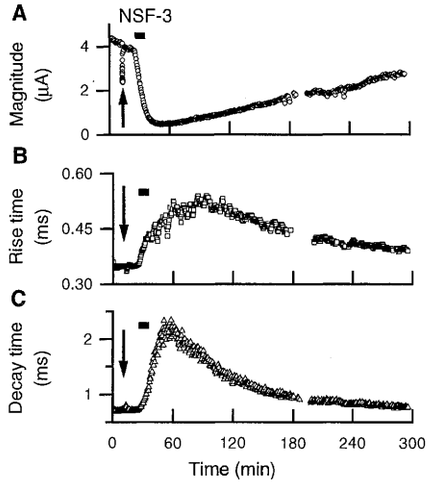

Fig. 5. Time courses of the physiological actions of NSF-3 injection (black bar) on EPSC amplitude (**A**), 20 to 80% rise time (**B**), and the time constant of a single-exponential function fit to EPSC decay (**C**). Arrow indicates responses to a train of presynaptic stimuli (1 Hz, 30 s), which reduced the amplitude of EPSCs without affecting their kinetics..

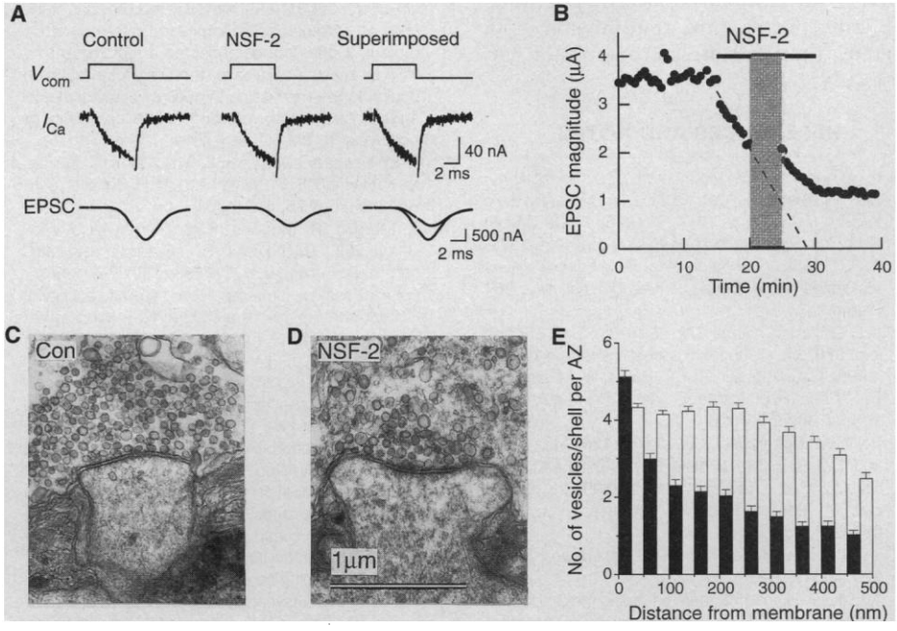

Fig. 6. Site of action of NSF peptides. (**A**) Lack of effect of NSF peptides on Ca^{2+} currents. Presynaptic Ca^{2+} currents (I_{Ca}) (middle) and EPSCs (bottom) elicited by presynaptic depolarization [from a command potential (V_{com}) of −60 to −22 mV for 6 ms] (top) before (control) and during (NSF-2) injection of NSF-2. Traces are shown superimposed on the right. (**B**) Lack of effect of NSF-2 injection (black bar) on release when stimulation was interrupted (shaded areas), indicating use-dependent inhibition. (**C** and **D**) Electron micrographs of a control terminal injected with buffer only (C) and of a terminal injected with NSF-2 that was fixed when the peptide had reduced EPSC amplitude by 91% (D). (**E**) Distribution of synaptic vesicles per active zone (AZ) at increasing distance (shell size, 50 nm) from the presynaptic plasma membrane for control terminals (white bars, $n = 520$ AZs, three terminals injected with scNSF-2, NSF-4, or buffer only) and those injected with NSF-2 (black bars, $n = 206$ AZs, two terminals).

both endocytosis and exocytosis.

Because NSF peptides both slow release kinetics and inhibit the ATPase activity of NSF, ATP hydrolysis likely results in conformational changes (*25*, *27*) that activate SNARE proteins (*28*) to promote membrane fusion in response to Ca^{2+} entry. Our results are consistent with a role for NSF before fusion in a reaction analogous to the ATP-dependent "priming" that precedes fusion of docked vesicles in endocrine cells (*23*, *29*, *30*). Alternatively, the NSF peptides might retard vesicle fusion by preventing NSF from dissociating SNARE complexes after fusion (*25*). However, regardless of when in the vesicle life cycle NSF exerts its action (or actions), the consequences of this action are apparent during the membrane fusion reaction itself. The kinetic changes induced by NSF peptides might result from slowing of a reaction that causes each synaptic vesicle to fuse [for example, dilation of a fusion pore (*31*)] or from desynchronization of individual vesicle fusion events (*32*). Desynchronization of vesicle fusion could result from a reduction in the number of fusogenic particles per vesicle, with inhibition occurring when the number of particles has been reduced to zero (*33*). Incorporating such temporal control into the basic mechanism of membrane fusion provides an economical means of defining the rate of synaptic transmission and, thereby, the integrative properties of synapses (*34*).

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7. An Eco RI–Hind III fragment encoding the D1 domain of CHO cell NSF (*4*) was labeled with ^{32}P (Rediprime, Amersham) and used to screen a squid optic lobe λgt10 library as described (*19*) with a hybridization and washing temperature of 50°C. One of five independent squid NSF clones containing the largest complement of coding sequence was sequenced (Sequenase, Applied Biosystems). This clone contains the complete coding sequence of a squid NSF protein with the exception of a 5′ sequence expected to encode ~140 NH_2-terminal amino acids (GenBank accession number Y16416).
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9. Electrical measurements and microinjections were performed on isolated stellate ganglia of *Loligo pealei* [G. J. Augustine and R. Eckert, *J. Physiol. (London)* **346**, 257 (1984)]. Unless otherwise indicated, transmitter release was evoked by presynaptic action potentials elicited once every 30 s. Experiments were performed in external saline with normal (11 mM) or reduced (5.5 mM) external Ca^{2+} concentrations with equivalent results. Two-electrode voltage-clamp recording was performed with Axoclamp 2A or Geneclamp 500 amplifiers (Axon Instruments, Foster City, CA). For measurement of postsynaptic currents, current-passing and voltage-measuring electrodes contained 7 M CsCl and 3 M KCl, respectively. When measuring presynaptic Ca^{2+} currents, tetraethylammonium chloride was injected to block K^+ currents; Na^+ and residual K^+ currents were then blocked by bath application of 100 μM tetrodotoxin (Calbiochem) and 2 mM 3,4-diaminopyridine (Sigma). The current-passing electrode contained injection buffer (*8*) and was used for microinjections. Presynaptic leak currents were obtained by averaging 10 hyperpolarizing pulses of one-fifth the amplitude of the depolarizing voltage step. Data were filtered at 3 to 10 kHz, digitized at 33 kHz, and analyzed with software written in Axobasic. Single-exponential fits to the decay of EPSCs starting at 80% of the peak amplitude were performed with the Simplex algorithm. Fluorescence images were acquired with an integrating charge-coupled device camera (Cohu, San Diego, CA) and analyzed with Imaging Workbench software (Axon Instruments). Peptides (acetylated at the NH_2-terminus and amidated at the COOH-terminus) were synthesized by Biosynthesis (Lewisville, TX), Chiron Mimotopes (Sidney, Australia), and the Protein Core Facility of the University of Texas Health Science Center (San Antonio, TX).
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14. For the assay of stimulation of the ATPase activity of recombinant six-histidine–tagged NSF-MYC by recombinant α-SNAP, Eppendorf tubes that had been coated with 20 μl of α-SNAP (100 ng/μl) were treated for 5 min with 12.5 μl of peptide solution (at four times the final concentration) before adding 37.5 μl of recombinant NSF (200 ng) in an ATPase assay buffer containing 25 mM tris-HCl (pH 7.4), 100 mM KCl, 0.5 mM dithiothreitol, 2 mM $MgCl_2$, and 0.6 mM ATP. After incubation for 45 min at 37°C, released phosphate was quantified with a colorimetric assay, and SNAP-stimulated ATPase activity was calculated by subtracting values obtained from parallel incubations containing no immobilized SNAP.
15. For testing postsynaptic responsiveness to glutamate, 100 μM caged glutamate [*N*-(α-carboxy-2-nitrobenzyl)-L-glutamic acid] (Molecular Probes) was added to the bath solution. Flashes of ultraviolet light were delivered through a 20× water immersion lens (numerical aperture, 0.5) (Olympus) with a capacitor discharge flash lamp (Cairn Research, Faversham, UK) [J. E. Corrie *et al.*, *J. Physiol. (London)* **465**, 1 (1993); R. Wieboldt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8752 (1994)].
16. Differences in the time courses of peptide disruption of release kinetics and EPSC amplitude evident in Fig. 4A were not observed in all experiments, so it is unclear whether these two physiological actions arose from one or more molecular perturbations.
17. L-*trans*-Pyrrolidine-2,4-dicarbocylic acid (0.5 to 1 mM) and D,L-*threo*-β-hydroxyaspartic acid (300 to 600 μM) were tested in combination at 16° to 20°C [J. S. Isaacson and R. A. Nicoll, *J. Neurophysiol.* **70**, 2187 (1993); G. Tong and C. E. Jahr, *Neuron* **13**, 1195 (1994); J. S. Diamond and C. E. Jahr, *J. Neurosci.* **17**, 4672 (1997)].
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30. Although NSF has been proposed to act before docking in yeast vacuolar fusion (*24*), it is possible that the "predocking" reaction monitored in such in vitro studies is equivalent to the priming observed here and elsewhere (*23*, *29*). For example, docking of synaptic vesicles might lead to a biochemically labile vesicle-membrane interaction that is consolidated during subsequent NSF-regulated priming.
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