FEBS Letters 435 (1998) 84–88 FEBS 20774

The 26-mer peptide released from SNAP-25 cleavage by botulinum neurotoxin E inhibits vesicle docking

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Received 3 August 1998

Abstract Botulinum neurotoxin E (BoNT E) cleaves SNAP-25 at the C-terminal domain releasing a 26-mer peptide. This peptide product may act as an excitation-secretion uncoupling peptide (ESUP) to inhibit vesicle fusion and thus contribute to the efficacy of BoNT E in disabling neurosecretion. We have addressed this question using a synthetic 26-mer peptide which mimics the amino acid sequence of the naturally released peptide, and is hereafter denoted as ESUP E. This synthetic peptide is a potent inhibitor of Ca^{2+}-evoked exocytosis in permeabilized chromaffin cells and reduces neurotransmitter release from identified cholinergic synapses in in vitro buccal ganglia of *Aplysia californica*. In chromaffin cells, both ESUP E and BoNT E abrogate the slow component of secretion without affecting the fast, Ca^{2+}-mediated fusion event. Analysis of immunoprecipitates of the synaptic ternary complex involving SNAP-25, VAMP and syntaxin demonstrates that ESUP E interferes with the assembly of the docking complex. Thus, the efficacy of BoNTs as inhibitors of neurosecretion may arise from the synergistic action of cleaving the substrate and releasing peptide products that disable the fusion process by blocking specific steps of the exocytotic cascade.

Key words: SNARE hypothesis; Neurosecretion; Exocytosis; Synaptic transmission; Protein-protein interaction

1. Introduction

A widely held view considers that the process of vesicle fusion with the plasma membrane which occurs during neuronal exocytosis is mediated by SNARE proteins [1–5]. This family of membrane proteins provides a specific means of pairing vesicles (v-SNAREs) with target (t-SNAREs) membranes [1–5]. *Clostridial* neurotoxins are metalloproteases that cleave specific components of the v-SNARE and t-SNARE and abolish neurotransmitter release. Botulinum neurotoxins (BoNT) B, D, F, and G, and the structurally related tetanus toxin specifically cleave VAMP at different sites [6,7]; BoNT A and E cleave SNAP-25 at the C-terminus [8,9], and BoNT C cuts syntaxin and SNAP-25 [10,11]. Proteolysis of each of these substrates produces a truncated protein and releases a peptide product [6–11]. It has been proposed that these peptide products may also prevent the formation of the core complex and thereby abrogate Ca^{2+}-triggered exocytosis [12,13]. This hypothesis is supported by the finding that truncated fusion proteins and synthetic peptides that mimic the amino acid sequence of segments from synaptotagmin [14,15], SNAPs [16], synaptobrevin [17], syntaxin [18], Ca^{2+} channels [19], and SNAP-25 [12,13,20] are specific inhibitors of neurosecretion. In particular, a 20-mer peptide encompassing the C-terminal domain of SNAP-25 blocked exocytosis by inhibiting vesicle docking in permeabilized chromaffin cells [12,13]. The term ESUP (excitation-secretion uncoupling peptide) was coined to highlight this inhibitory activity [12]. Although these results suggest that peptide products resulting from substrate cleavage by BoNTs may block vesicle fusion, experimental support to substantiate this notion is still limited.

Here, we show that a 26-mer peptide corresponding to the amino acid sequence of the peptide product released by BoNT E cleavage of SNAP-25, referred to as ESUP E, efficiently and selectively blocks Ca^{2+}-evoked exocytosis in chromaffin cells and neurotransmitter release in *Aplysia* cholinergic synapses. Our results are consistent with the notion that ESUP E prevents vesicle docking by interfering with the assembly of the synaptic ternary complex formed by SNAP-25, VAMP and syntaxin.

2. Material and methods

2.1. Reagents

[^{3}H]Noradrenaline was from DuPont-NEN (Boston, MA). t-Boc and Fmoc amino acids, with standard side chain protecting groups, were obtained from Applied Biosystems (Foster City, CA), NovaBiochem (La Jolla, CA) or Peninsula Laboratories (Belmont, CA). Solvents, reagents and resins for peptide synthesis were obtained from Applied Biosystems (Foster City, CA), Percoll from Pharmacia, collagenase (EC 3.4.24.3) from Boehringer Mannheim (Germany), anti-SNAP-25 mAb (clone SM81) from Sternberger (Baltimore, MD), anti-syntaxin mAb (clone HPC1) from Sigma (St. Louis, MO) and anti-VAMP Ab from Stressgen (Canada). Agarose-conjugated protein G was from Pierce (Rockford, IL). BoNTs were kindly provided by Drs. B.R. DasGupta and M. Goodnough (University of Wisconsin). All other reagents were of analytical grade from Sigma.

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Abbreviations: BoNT, botulinum neurotoxin; SNAP-25, synaptosomal associated protein of 25 kDa; ESUP, excitation-secretion uncoupling peptide; VAMP, vesicle associated membrane protein; SNARE, SNAP receptor; v-SNARE, vesicle-SNARE; t-SNARE, target-SNARE; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; IPSC, inhibitory postsynaptic current; ACh, acetylcholine

PII: S0014-5793(98)01012-6

2.2. Peptide synthesis and purification

ESUP E (SNAP-25 [181–206]: IMEKADSNKTRIDEANQRATKMLGSG) and ESUP E^{RDM} (ESDNDTRAIKITQAGSMKRMGLNAKE) were synthesized by Fastmoc Fmoc chemistries in an Applied Biosystems 431A automated solid-phase peptide synthesizer, cleaved and purified as described [12,13].

2.3. Activation of BoNT E

The single chain BoNT E in 25 mM HEPES was converted to the 'nicked' di-chain form by treatment with 0.3 mg/ml trypsin XI for 30 min at 37°C, followed by incubation with 0.5 mg/ml soybean trypsin inhibitor for 15 min at room temperature. Aliquots of the nicked toxin were frozen at −80°C, then thawed and treated with 1 mM dithiothreitol (DTT) immediately before use to expose the active site of the light chain protease.

2.4. Chromaffin cell cultures and secretion assays

Chromaffin cell cultures were prepared from bovine adrenal glands by collagenase digestion and further separated from debris and erythrocytes by centrifugation on Percoll gradients as described [12,13]. Cells were maintained in monolayer cultures at a density of 625 000 cells/cm^2 and were used 3–6 days after plating. All the experiments were performed at 37°C. Secreted [^{3}H]noradrenaline was assayed in digitonin-permeabilized cells as described [12,13]. The CPM released from control cells under basal conditions was ∼3000, and increased to ∼11 000 when stimulated with 10 μM Ca^{2+}. The total number of counts obtained from detergent-permeabilized cells was ∼110 000. Thus, the normalized basal release represents 3.5% of the total secretion, and the Ca^{2+}-evoked component accounts for ∼10% of the total. Statistical significance was calculated using Student's *t*-test with data from ≥4 independent experiments.

2.5. Immunoprecipitation of the ternary complex SNAP-25/VAMP/syntaxin from solubilized rat brain synaptosomes

Rat brain synaptosomes were prepared from brain cortices as described [21]. Synaptosomes (100 μg) were solubilized in radioimmunoprecipitation assay buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% deoxycholate, 1 mM EGTA, 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and 5 mM iodoacetamide), incubated with or without 100 μM ESUPs for 2 h at 4°C, unless otherwise indicated. Insoluble material was removed by centrifugation at 10 000 × *g* for 30 min at 4°C. Immunopurification of the ternary complex SNAP-25/VAMP/syntaxin from the soluble material was achieved by using an overnight incubation with anti-SNAP-25 monoclonal antibody (1 μg mAb/100 μg protein). Immunocomplexes were captured with agarose-conjugated protein G (100 μl, 50% slurry), and washed six times with 500 μl of radioimmunoprecipitation buffer at 4°C. Immunoprecipitates were dissolved with 50 μl of SDS-PAGE buffer, boiled 5 min, separated by SDS-PAGE and analyzed by immunoblotting. Blots were probed with the anti-SNAP-25 mAb, an anti-syntaxin mAb and an anti-VAMP Ab. Bands were visualized using the ECL system, and quantified using the public domain NIH Image program version 1.57 [13]. Data are given as mean ± S.E.M., with *n* (number of experiments) = 3.

2.6. Inhibition of neurotransmitter release in Aplysia synapses

Experiments were performed with neuronal preparations from the marine mollusc *Aplysia californica.* Intraneuronal inhibition of nerve-evoked release of acetylcholine (ACh) was measured at identified cholinergic synapses of *Aplysia* buccal ganglia [22,23]. The ganglia were surgically removed and pinned to the Sylgard lined bottom of an acrylic chamber, and the connective tissue capsule was excised. The soma of identified pre- and postsynaptic cholinergic neurons were impaled with glass microelectrodes (2–4 MΩ) filled with 2 M potassium acetate. Action potentials were evoked in presynaptic neurons by suprathreshold depolarizing stimuli applied at 0.1 Hz. Neurotransmitter release was assessed by measuring the amplitudes of inhibitory postsynaptic currents (IPSCs) in voltage-clamped follower neurons. Presynaptic potentials and postsynaptic currents were digitized and stored on a personal computer using pClamp software (Axon Instruments, Foster City, CA). Only responses that were not accompanied by spontaneous activity were analyzed. The preparation was superfused continuously at a rate of 1 ml/min with artificial sea water containing in mM: NaCl 480; KCl 10; $CaCl_2$ 10; $MgCl_2$ 20; $MgSO_4$ 30; $NaHCO_3$ 2.5; HEPES 10, at pH 7.8, maintained at room temperature. ESUP E and ESUP E^{RDM} (5 mM) were dissolved in 600 mM NaCl containing 1% (w/v) fast green FCF dye to aid in visualizing the volume injected. The solution was air pressure-injected into the presynaptic cell by micropipette. A maximum pressure of 60 psi was used to introduce an adequate volume of solution into the presynaptic cell as indicated by the appearance of intracellular dye. The volume of solution injected was ≤10% of the estimated cell volume, yielding a final intracellular peptide concentration ≤100 μM. BoNT E concentration in the micropipette was 3.3 μM.

3. Results and discussion

3.1. A peptide mimicking the 26-aa peptide fragment released by BoNT E cleavage of SNAP-25 blocks exocytosis

Cleavage of the C-terminus of SNAP-25 by BoNT E releases a 26-mer peptide that may block neurosecretion [12,13]. To test this hypothesis, we synthesized this 26-mer peptide (ESUP E) and assayed the presumed inhibitory activity on Ca^{2+}-evoked catecholamine release from digitonin-permeabilized chromaffin cells. ESUP E blocked noradrenaline

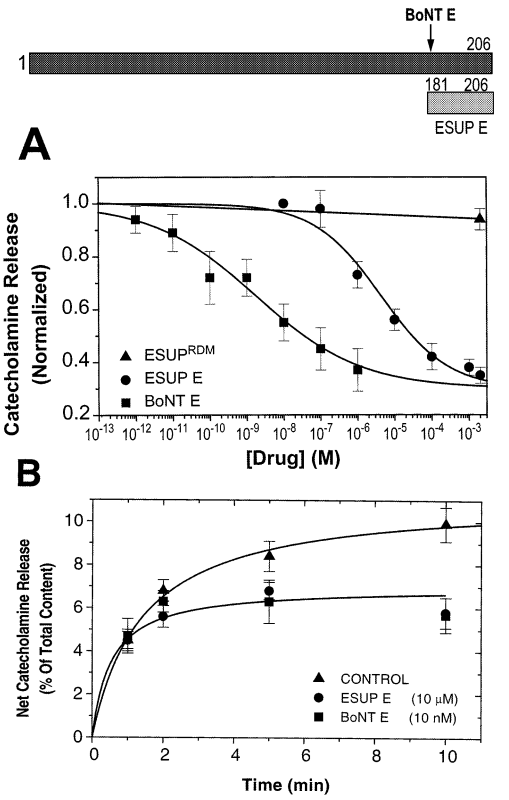

Fig. 1. ESUP E blocks Ca^{2+}-dependent catecholamine secretion from permeabilized chromaffin cells. Top: Schematic representation of SNAP-25 with the cleavage site for BoNT E. ESUP E represent the peptide product (aa 181–206) released by BoNT E cleavage of SNAP-25. Bottom: A: Concentration-dependent inhibition of Ca^{2+}-evoked catecholamine release from pemeabilized chromaffin cells by BoNT E, ESUP E and ESUP E^{RDM}. Net release is given as mean ± S.E.M. with *n* (number of experiments performed in triplicate) = 4. Solid lines depict the best fit to the logistic equation: $B/B_{max} = 1/(1+([blocker]/IC_{50})^n)$, where B denotes the extent of block, B_{max} represents the maximal block; IC_{50} denotes the concentration of blocker (BoNT E or ESUP E) that produces half-maximal block, and n is the Hill coefficient of the blocking activity. For BoNT E the values were $IC_{50} = 1.8 \pm 1.3$ nM, n = 0.5; and for ESUP E, $IC_{50} = 250 \pm 75$ nM, n = 0.6. B: Time course of the net noradrenaline release (Ca^{2+}-stimulated minus basal) obtained in presence or absence of 100 μM ESUP E or 10 nM BoNT E.

release with an IC_{50}=250 ± 75 nM, and a maximal inhibition of ~70% (Fig. 1A). The ESUP inhibitory activity was similar to that elicited by BoNT E with respect to maximal inhibition (~70%) but was ~140-fold less efficient (BoNT E IC_{50}=1.8 ± 1.3 nM). The sequence specificity of ESUP activity was assessed by synthesizing a randomized version of the peptide (ESUP E^{RDM}), which was proven inert in blocking catecholamine release at concentrations up to 100 μM (Fig. 1A).

To identify the step of the exocytotic cascade blocked by ESUP E, we investigated its activity on the kinetics of the secretory process and compared it with that produced by BoNT E (Fig. 1B). Permeabilized cells were incubated with ESUP E or DTT-reduced BoNT E for 5 min, and secretion was evoked by Ca^{2+} pulses of different duration. Incubation of permeabilized chromaffin cells with 10 μM ESUP E or 10 nM BoNT E inhibited ~60% of catecholamine release, primarily by altering the slow phase of secretion (Fig. 1B), suggesting that the vesicle pools upstream of docking and priming steps are sensitive to the action of ESUP E and BoNT E [24–26]. These data indicate that the 26-mer peptide released by BoNT E cleavage of SNAP-25 is a potent and specific uncoupler of Ca^{2+}-evoked exocytosis, and suggest that the efficiency of BoNT E to disable the fusion process may arise from the combined action of cleaving a protein critical for the assembly of the fusion complex, and by releasing a small peptide which, in turn, may interfere with the formation of the complex.

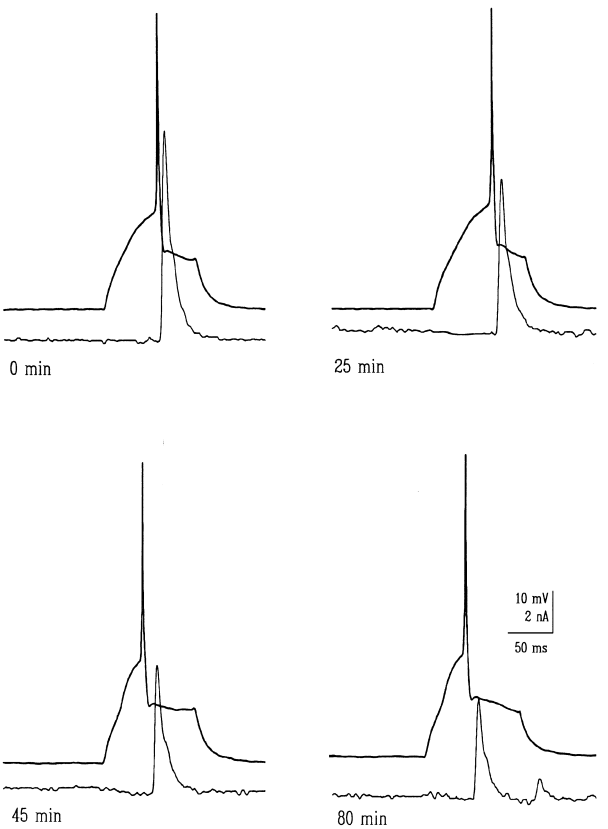

Fig. 2. ESUP E blocks ACh release by the presynaptic neuron in a cholinergic synapse in *Aplysia* buccal ganglia. ACh release was monitored as the amplitude of the IPSC (lower trace) elicited by an evoked action potential (upper trace) in the presynaptic neuron. Recordings show the decrement of the IPSC amplitude after injection of ESUP E into the presynaptic neuron at zero time.

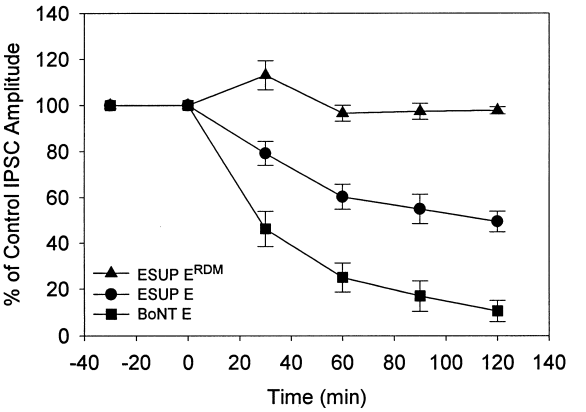

Fig. 3. ESUP E and BoNT E inhibit IPSC amplitude in *Aplysia* buccal ganglion synapses. Active ESUP E (n = 10), the inactive random sequence ESUP E^{RDM} analog (n = 8), or BoNT E (n = 7) were injected into the presynaptic neuron at zero time. IPSC amplitude was inhibited after injection of active ESUP E and BoNT E but not by injection of the inactive analog. Mean ± S.E.M.

3.2. *ESUP E and BoNT E inhibit neurotransmitter release in Aplysia cholinergic synapses in vitro*

Release of ACh by the presynaptic neuron in response to electrically evoked action potentials was assessed from the amplitudes of the evoked IPSCs in a voltage-clamped postsynaptic neuron. Fig. 2 shows superimposed action potentials and IPSCs in a typical experiment. ESUP E was injected into the presynaptic neuron at zero time (top left panel), and the resultant decline of IPSC amplitude is shown at three successive time points. The IPSC amplitude declined to 52% of the control value 120 min after the injection of ESUP E. The decrease of IPSC amplitude was gradual and incomplete, typically requiring 2 h to reach a stable value of 30–70% of the control. The time course of the effect of peptides or toxin injection on IPSC amplitude is shown in Fig. 3. BoNT E, ESUP E or ESUP E^{RDM} were injected at time zero. The increase of IPSC amplitude immediately following the injection of ESUP E^{RDM} was not considered to be significant since such increases were a frequent consequence of pressure injection of any compound, and IPSC amplitudes typically returned to control values within 20 min. No further reduction of responses occurred in cells injected with the random-sequence control peptide, whereas IPSCs in cells injected with BoNT E and active ESUP E declined to a stable level over the ensuing 120 min.

The rate and extent of IPSC inhibition caused by BoNT E was greater than that produced by ESUP E: the amplitude was attenuated by 50% in 28 min and by 90% in 120 min by BoNT E, whereas inhibition by ESUP E was only 50% at 120 min. The more rapid and nearly complete IPSC decrement caused by BoNT E supports the concept that the inhibition caused by the toxin protease is a consequence of both a decrease in available SNAP-25 and an accumulation of cleavage products.

3.3. *ESUP E inhibits vesicle docking by interfering with the formation of the ternary complex comprising SNAP-25, VAMP, and syntaxin*

Since the C-terminal domain of SNAP-25 binds tightly to VAMP and syntaxin during vesicle docking, forming a highly stable ternary complex, it is conceivable that ESUP E blocks

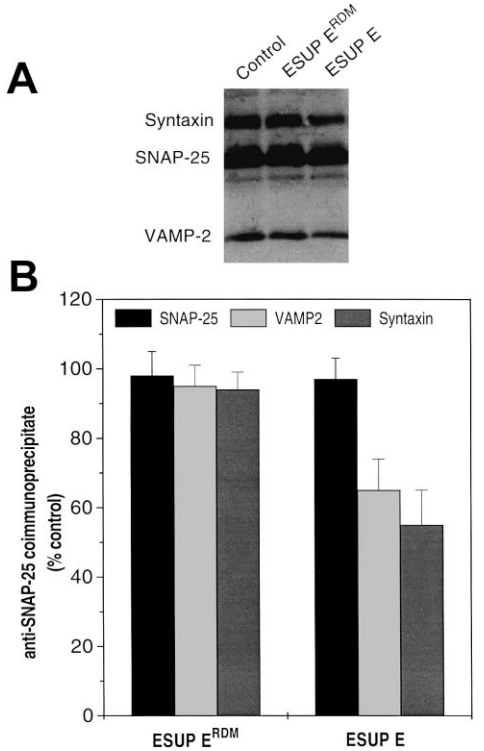

Fig. 4. ESUP E disrupts the interaction between SNAP-25, VAMP and syntaxin. A: Immunoprecipitates of the ternary complex SNAP-25/VAMP/syntaxin from rat brain synaptosomes incubated without (Control) or with 100 μM ESUP E or ESUP E^{RDM}. Immunocomplexes were analyzed using SDS-PAGE (4–20%) under non-reducing conditions, and immunoblotted with an anti-syntaxin mAb, anti-SNAP-25 mAb and anti-VAMP Ab. B: Data of three different experiments were quantified by image analysis, and values normalized with respect to that of control.

the docking process by competition with SNAP-25 for binding to the ternary complex. To examine this question, we studied the effect of ESUP E on the interaction of SNARE proteins in digitonin-permeabilized chromaffin cells. We did not observe coimmunoprecipitation of all the SNARE proteins with the anti-SNAP-25 mAb (data not shown), as reported by others [27]. We therefore turned to rat brain synaptosomes as an alternative preparation [3,20,28]. Since the SNARE complex forms spontaneously during synaptosome preparation and solubilization, we evaluated if ESUP E could displace SNAP-25 from the ternary complex and, therefore, dissociate the preformed aggregate or interfere with its assembly. The experimental protocol involved detergent solubilization of synaptosomes, incubation with ESUP E or ESUP E^{RDM}, immunopurification of the ternary complex using an anti-SNAP-25 mAb followed by separation of the components using SDS-PAGE. Immunoblots probed with specific antibodies raised against syntaxin, SNAP-25 and VAMP revealed the presence of the three proteins in the immunoprecipitate (Fig. 4A). Incubation with 100 μM ESUP E inhibited the coimmunoprecipitation of VAMP and syntaxin without affecting the immunopurification of SNAP-25.

A quantitative analysis of the immunoblots is shown in Fig. 4B. An excess of ESUP E inhibited the coimmunoprecipitation of VAMP by ~30% and of syntaxin by ~40%, whereas no effect was detected with ESUP E^{RDM}, in accord with expectations. The partial inhibition produced by ESUP E (100 μM) may be accounted for by its relatively low affinity (Fig. 1A). Nonetheless, the fact that a short peptide may interfere with the assembly or stability of an SDS-resistant complex is highly significant, and provides experimental support for the notion that ESUP E inhibits vesicle docking by preventing the formation of the essential ternary complex. These findings suggest that ESUP E may compete with SNAP-25 for binding to VAMP and interrupt the ensuing chain of protein-protein interactions that lead to vesicle fusion.

3.4. Molecular mechanism of ESUPs biological activity

The finding that the 26-mer peptide released from SNAP-25 cleavage by BoNT E mimics the inhibitory action of this neurotoxin on neurosecretion (Fig. 1), and on synaptic transmission (Figs. 2 and 3), provides support to the tenet that BoNTs abrogate vesicle fusion by the combined action of cleaving the substrate and releasing peptide products which block the docking or/and priming steps of the exocytotic cascade. The result that the ternary complex is specifically disrupted by an excess of ESUP E (Fig. 4) supports this view. The fact that the 20-mer ESUP A (SNAP-25 [187–206]: SNKTRIDEANQRATKMLGSG), corresponding to the C-terminal sequence of SNAP-25, arrests the ATP-dependent maturation of the secretory granules and promotes the accumulation of secretory vesicles near the plasma membrane is in accord with this notion [13]. Recent studies implicate the C-terminal segment of SNAP-25 encompassing residues 180–196 in vesicle docking and in a late post-docking step [29,30]. Our finding that ESUP E is a more efficient inhibitor of neurosecretion than ESUP A supports this conclusion. Thus, ESUPs mimicking specific protein domains provide novel tools to dissect their contribution to different steps of neurosecretion.

Acknowledgements: We thank B. R. DasGupta and M. Goodnough for purified BoNT E. This work was supported by Grants from the Spanish DGICYT PM-0110 (to S.V.), the US Army Medical Research and Materiel Command DAMD 17-93-C-3100 and DAMD 17-98-C-8040 (to M.M.), and a Postdoctoral Fellowship from the Dystonia Medical Research Foundation (to J.M.C.).

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