

## Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds

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SNAP-25, a membrane-associated protein of the nerve terminal, is specifically cleaved by botulinum neurotoxins serotypes A and E, which cause human and animal botulism by blocking neurotransmitter release at the neuromuscular junction. Here we show that these two metallo-endopeptidase toxins cleave SNAP-25 at two distinct carboxyl-terminal sites. Serotype A catalyses the hydrolysis of the Gln<sup>197</sup>–Arg<sup>198</sup> peptide bond, while serotype E cleaves the Arg<sup>180</sup>–Ile<sup>181</sup> peptide linkage. These results indicate that the carboxyl-terminal region of SNAP-25 plays a crucial role in the multi-protein complex that mediates vesicle docking and fusion at the nerve terminal.

Botulism; Neuroexocytosis; SNAP-25; VAMP; Neurotoxin; Proteinase

### 1. INTRODUCTION

The flaccid paralysis of botulism is caused by a structurally related family of botulinum neurotoxins (BoNT), composed of seven different serotypes (A,B,C,D,E,F, and G) [1–3]. BoNTs penetrate motor-neurons at the neuromuscular junction where they block acetylcholine release. Recent studies have demonstrated that these toxins are zinc-dependent endopeptidases and that their effect on neurotransmission is mediated through the selective cleavage of proteins of the presynaptic terminal thought to be involved in docking and fusion of synaptic vesicles [4,5].

These neurotoxins exhibit both conformational as well as sequence specificity. Thus tetanus neurotoxin (TeNT) and BoNT/B, /D and /F have each been shown to cleave the synaptic protein VAMP/synaptobrevin at a single site that differs between these toxin serotypes [4–10]. More recently, BoNT/A and /E were shown to recognize and cleave SNAP-25 (synaptosomal-associated protein of 25 kDa) [11,12]. Unlike the vesicle-associated VAMP, SNAP-25 is located at the plasma mem-

brane [13–15]. Söhlner et al. [16] have shown that both SNAP-25 and VAMP are components of a 20S multi-protein complex proposed to mediate vesicle docking and fusion. SNAP-25 also appears to be required for axonal growth during development and possibly nerve terminal plasticity in the mature nervous system [17,18]. However, with the exception of sites for fatty acid acylation [15], regions of the SNAP-25 polypeptide chain that mediate these intermolecular interactions have not been identified.

Here we show that these two neurotoxins hydrolyse two different peptide bonds located in the COOH-terminal region, suggesting that this part of SNAP-25 is of critical importance for vesicle docking and fusion.

### 2. MATERIALS AND METHODS

#### 2.1. *Proteins and chemicals*

BoNT/A and /E were isolated and purified to remove traces of contaminant proteases as before [19,20]. Their serotype identity was assessed by the different electrophoretic mobility [21] and by five steps of amino acid sequencing. Recombinant SNAP-25 was prepared by subcloning the *Nco*I to *Bam*HI fragment bearing the entire protein coding region (717 bp) of the mouse cDNA plasmid clone p8.52 [13] downstream of the T7 RNA transcription and protein translation signals of the T7 PET expression vector, pET11d (Novagen). The SNAP-25 expression plasmid was used to transform *E. coli* strain BL21 DE3 and soluble extracts were prepared after induction of T7 RNA polymerase with IPTG. The recombinant SNAP-25 was collected as a 25–55% ammonium sulfate precipitate, dialysed and fractionated by FPLC-MonoQ column (Pharmacia) with a 20–500 mM NaCl gradient (Prashant P. Mehta, Jaime Jontes and Michael C. Wilson, in preparation). The recombinant protein was identified dur-

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**Abbreviations:** SNAP-25, synaptosomal-associated protein of 25 kDa; VAMP, vesicle associated membrane protein or synaptobrevin; BoNT/A, botulinum neurotoxin serotype A; BoNT/E, botulinum neurotoxin serotype E; TeNT, tetanus neurotoxin; FA-22.5, 22.5 kDa SNAP-25 BoNT/A-generated fragment; FE-20.5, 20.5 kDa SNAP-25 BoNT/E-generated fragment; DTT, dithiothreitol.

ing purification by immunoblotting with a specific polyclonal antiserum [13]. The protein was estimated to be 60–70% pure by Coomassie blue staining of SDS polyacrylamide gels. This was sufficient for the analysis of the pattern of neurotoxin proteolysis because no proteins of size lower than SNAP-25, which could interfere with the analysis, were present. Rabbit polyclonal antiserum against recombinant SNAP-25 was prepared as before [13] with a bacterial expressed recombinant fusion protein, corresponding to residues 33–206 of the SNAP-25 polypeptide sequence prepared as above. Prior to immunization, the recombinant SNAP-25 was partially purified by DEAE-Sephacel and Sephacryl S-200 (Pharmacia) chromatography (Richard A. Hart and Michael C. Wilson, unpublished results).

## 2.2. Proteolytic activity of botulinum neurotoxins A and E on synaptosomes and recombinant SNAP-25

BoNT/A and /E proteolysis of SNAP-25 in synaptosomes, isolated from rat brain cortex, was assayed as before [12]. BoNT proteolysis of recombinant SNAP-25 was performed as follows. Recombinant SNAP-25 in 50 mM Tris-HCl, 10 mM NaP<sub>i</sub>, 300 mM NaCl, 2 mM MgCl<sub>2</sub>, 0.3 mM CaCl<sub>2</sub>, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.25 NaN<sub>3</sub>, pH 7.6 was treated with reduced BoNT/A or /E (200 nM final concentration) for 2.5 hours at 37°C. Samples were made 6.5% in trichloroacetic acid, centrifuged and pellets were subjected to SDS polyacrylamide gel electrophoresis and stained as before [12]. Protein bands corresponding to the 22.5 kDa and 20.5 kDa SNAP-25 BoNT/A and /E-generated fragments were electroeluted, applied to ProSpin tubes and submitted to automatic Edman degradation with a gas-

phase sequencer (Applied Biosystems model 470A) connected on-line with a phenylthiohydantoin amino acid analyzer with a Control Data Module model 900A, programmed for five cycles of analysis. In parallel, trichloroacetic acid supernatants were lyophilized, dissolved in 100 µl of trifluoroacetic acid 0.05% and analyzed in Beckman Sistem Gold HPLC with a C18 Ultra-Sphere analytical column eluted with a 0–40% CH<sub>3</sub>CN/0.05% trifluoroacetic acid linear gradient. Peptide peaks were collected and sequenced for ten cycles.

## 3. RESULTS

### 3.1. SNAP-25 is cleaved by BoNT/A and BoNT/E at two distinct sites

SNAP-25 is a 206 residue nerve terminal protein, associated to the plasma membrane, possibly via palmitoyl chains linked to one or more of the four cysteine residues, located in the central region of the polypeptide chain [15]. As shown in Fig. 1A, BoNT/A and /E convert synaptosomal SNAP-25 (23.3 kDa), to lower molecular weight fragments, recognized by a polyclonal rabbit antiserum, raised against the recombinant fusion protein. While BoNT/A converts SNAP-25 to a 22.5 kDa fragment (FA-22.5), similar to that described by Blasi et al. [11], BoNT/E induces the formation of a 20.5

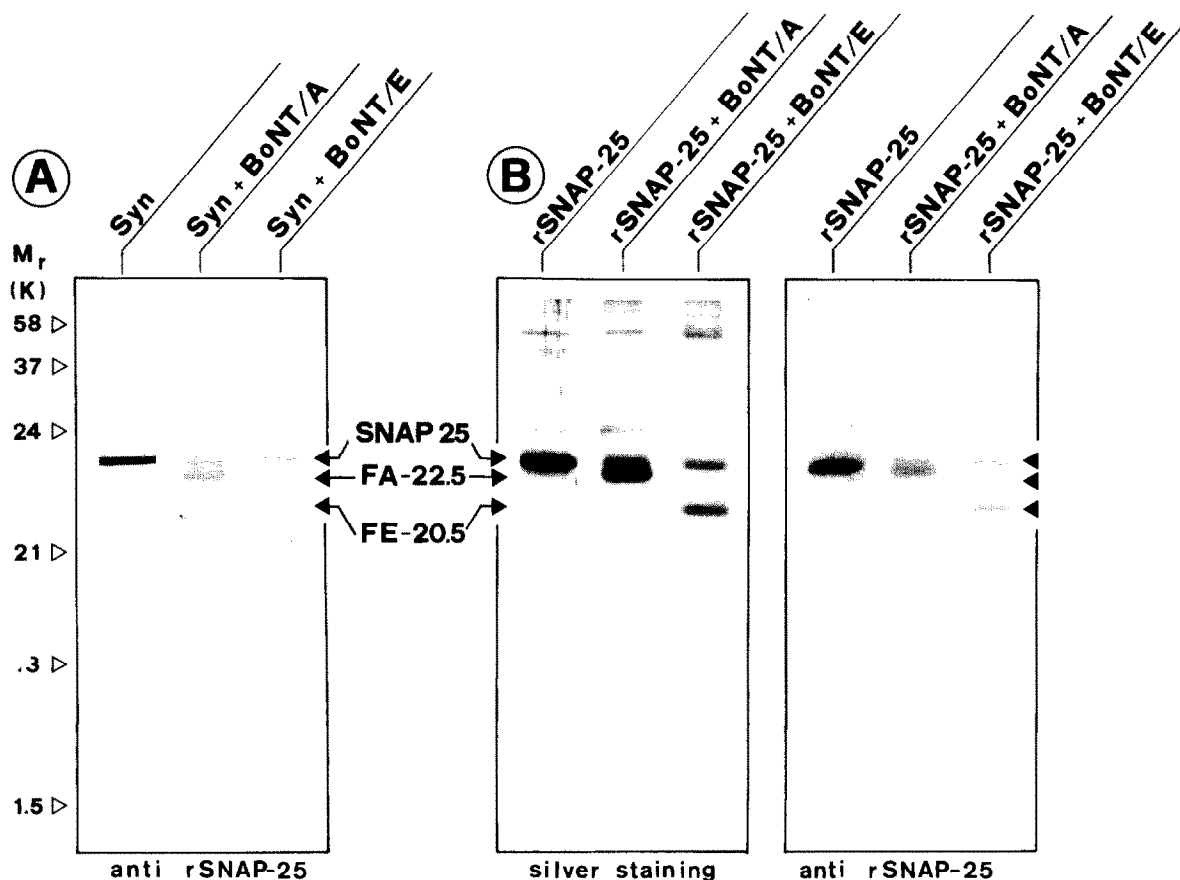


Fig. 1. Effect of BoNT/A and BoNT/E on synaptosomal and recombinant SNAP-25. (A) Rat brain synaptosomes were treated with buffer (Syn) or BoNT/A (Syn + BoNT/A) or BoNT/E (Syn + BoNT/E) as detailed in the experimental section. Samples were electrophoresed, blotted onto nitrocellulose and stained with a rabbit anti-recombinant SNAP-25 antiserum developed with anti rabbit alkaline phosphatase conjugated IgG. (B) Recombinant SNAP-25 (rSNAP-25) was incubated with buffer (rSNAP-25) or BoNT/A (rSNAP-25 + BoNT/A) or BoNT/E (rSNAP-25 + BoNT/E), electrophoresed as the samples of panel A and visualized by silver staining or as done for the samples of panel A.

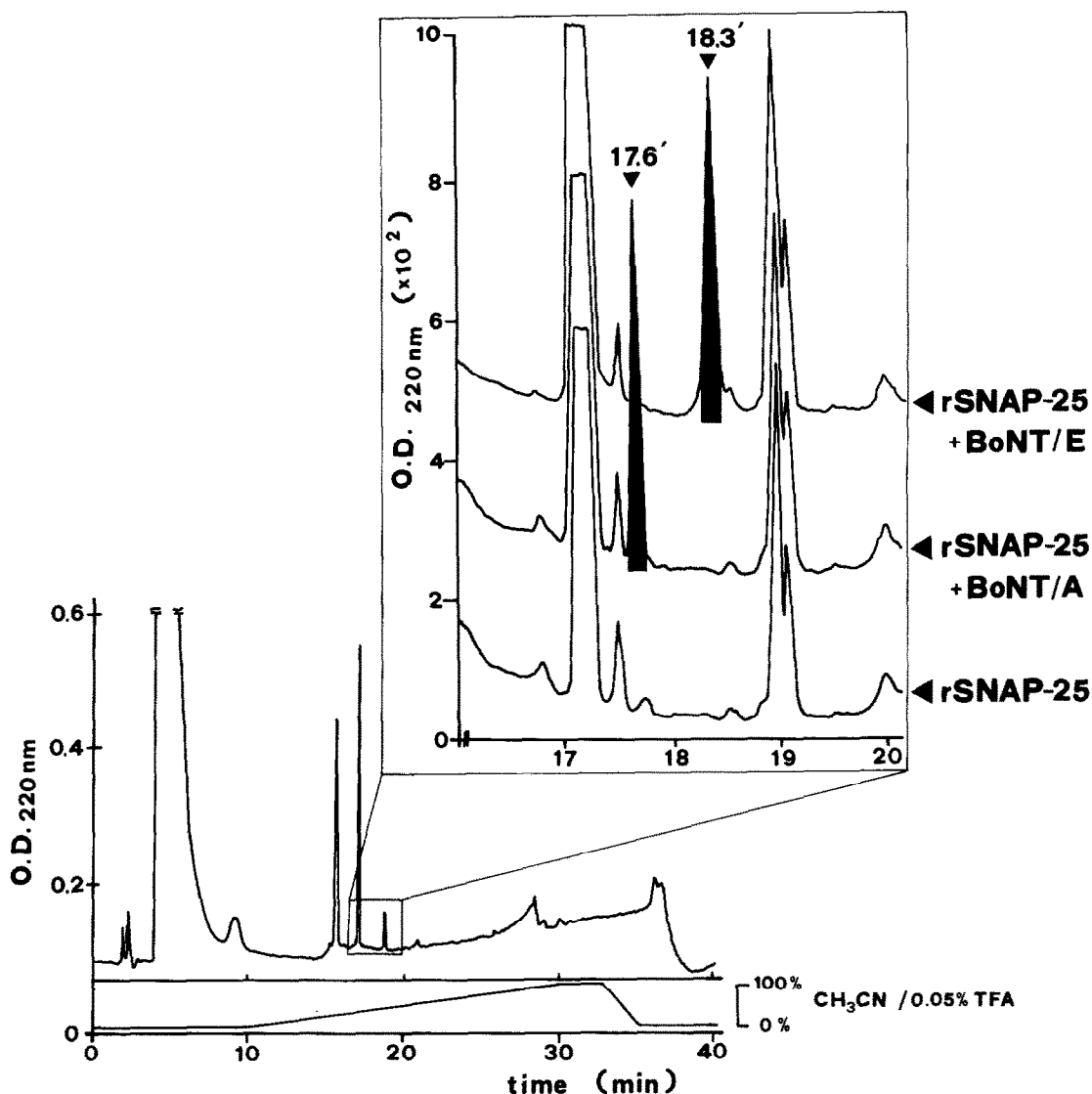


Fig. 2. Chromatographic analysis of the peptides generated by BoNT/A and BoNT/E proteolysis of recombinant SNAP-25. Recombinant SNAP-25 (rSNAP-25) was incubated with buffer or BoNT/A or BoNT/E, as the samples of Fig. 1, treated with trichloroacetic acid and the supernatants were subjected to chromatographic analysis with a linear  $\text{CH}_3\text{CN}/0.05\%$  trifluoroacetic acid gradient as described in the experimental section. The lower panel shows the chromatogram of recombinant SNAP-25 and the upper panel reports a  $10\times$  expansion of the region of retention times 16–20 min showing the peptide peaks appearing upon incubation with BoNT/A (retention time 17.6 min) or with BoNT/E (18.3 min). These peaks do not overlap to any of the peaks present in the buffer-treated rSNAP-25.

kDa fragment (FE-20.5). Since neither of these fragments are recognized by an antiserum raised against the twelve carboxyl-terminal residues of SNAP-25 [13], these results indicate that BoNT/A and /E cleave rat brain synaptosomal SNAP-25 at different sites, located in the 3 kDa COOH-terminal region of the protein. The small carboxyl-terminal fragments, however, were not detected by any of the available anti-SNAP-25 antisera, and nor we were able to identify them by Coomassie blue or silver staining, even after two-dimensional gel electrophoresis. These data, therefore, do not distinguish whether the toxins cleave SNAP-25 at single or multiple sites.

To discriminate between these possibilities, recombi-

nant SNAP-25 proteolysis by BoNT/A or BoNT/E was studied. Fig. 1B shows that, when recombinant SNAP-25 was incubated with BoNT/A or BoNT/E, fragments of electrophoretic mobility identical to those of FA-22.5 and FE-20.5 were generated. Interestingly, comparison of the silver staining and immunostaining profiles of SNAP-25 before and after treatment with the two neurotoxins indicates that the COOH-terminal residues of SNAP-25 form an immunodominant epitope and a minority of the antibodies of this serum recognize other epitopes. Since the recombinant protein serves as an efficient and accurately cleaved substrate, these data demonstrate that accessory neuronal factors, including fatty acylation or membrane attachment at the nerve

**FA-22.5**

2  
▼  
AEDAD

1 20 40 60 80 100  
▼ ▼ ▼ ▼ ▼  
MAEDADMRLNEEMQRRADQLADESLESTRRLQLVEESKDAGIRTLVMLDEQGEQLERIEEGMDQINKDMKEAEKNLTDLGKFCGLCVPCPNKLKSSDAYKK

2  
▼  
AEDAD

**FE-20.5**

Fig. 3. Amino acid sequence of mouse brain SNAP-25 and of the amino-terminal regions of the peptides generated by BoNT/A (above) and BoNT/E (below) determined by amino acid sequencing. The figure reports the c-DNA derived primary sequence of SNAP-25 [13] and the amino-terminal sequences of recombinant SNAP-25 fragments obtained as in Fig. 1. Recombinant SNAP-25 begins with Ala and the two BoNT generated fragments have the same amino-terminal sequence reported on the left part of the Figure. The sequences of the smaller fragments, isolated as shown in Fig. 2, are reported on the right.

terminal, are not necessary for the specific BoNT/A and /E proteolytic activity.

Previously, evidence that BoNT/B, /D, /F and TeNT hydrolyse a single peptide bond in their target VAMP/synaptobrevin, was provided by the cumulative estimated molecular weight of the two generated fragments [6,7,10]. Accordingly, silver staining of BoNT/E cleaved recombinant SNAP-25 revealed a faintly stained fragment ca. 3 kDa (undetectable after photographic printing) that together with the apparent 20.5 kDa mass of the larger fragment is consistent with the molecular weight of the intact protein (23.3 kDa). Unfortunately, the anticipated size of the COOH-terminal fragment produced by BoNT/A (ca. 1,000 Da, based on the decrease in  $M_r$  from 23.5 of SNAP-25 to 22.5 kDa of the larger fragment) would result in the expected fragment running in the dye front.

### 3.2. Isolation and sequencing of the SNAP-25 fragments generated by BoNT/A and BoNT/E hydrolysis

To identify the site of SNAP-25 proteolysis by BoNT/A and /E, the larger fragments, generated by digestion of the recombinant SNAP-25, were electroeluted from the polyacrylamide gel and sequenced as described previously [7,10,12]. As shown in Fig. 3, for both the BoNT/A and /E derived fragments, the identical sequence, AEDAD, was obtained. This corresponds to residues 2–6 of SNAP-25, as predicted from cDNA sequence [13]. The lack of the amino terminal Met residue is explained by the efficient excision of the initiating *N*-formyl-Met in *E. coli*, preferentially leaving penulti-

mate, short side chain residues (such as alanine) at the amino termini [22]. This confirms that the sites of BoNT/A and /E proteolysis are both located in the SNAP-25 carboxyl-terminal region.

To isolate the smaller toxin-generated SNAP-25 fragments, the SNAP-25-BoNT reaction mixture was acid precipitated, centrifuged and the clear supernatant was fractionated via HPLC over a C-18 column. The chromatograms reported in Fig. 2 show that fractionation of BoNT/A and /E-treated SNAP-25 resulted in the appearance of two peptide peaks with different retention times, reflecting cleavage products which were not present in the intact recombinant SNAP-25 preparation. The finding of only one additional peak in the BoNT/E-treated SNAP-25 samples indicates that this toxin does not cleave additionally at the site of BoNT/A proteolysis. However, BoNT/E still recognizes and cleaves SNAP-25 after BoNT/A proteolysis, as shown by the appearance in the chromatogram of a peptide peak of retention time intermediate between those of Fig. 2 (not shown).

Fig. 3 reports the sequences of the peptides, isolated as shown in Fig. 2. RATKMLGSG corresponds to the nine carboxyl terminal residues of SNAP-25 and was obtained from the BoNT/A-induced small SNAP-25 fragment. This sequence comprises a 920 Da peptide that would precisely account for the reduction of size of SNAP-25 caused by BoNT/A (Fig. 1), indicating that BoNT/A cleaves SNAP-25 at a single site, identified here as the Gln<sup>197</sup>–Arg<sup>198</sup> peptide bond. In contrast, the sequence IMEKADSNKT was determined from the

3 kDa BoNT/E-derived fragment, indicating that this serotype hydrolyzes the Arg<sup>180</sup>-Ile<sup>181</sup> peptide bond. The calculated molecular weight of the 181-206 SNAP-25 segment (2,865 Da), well accounts for the decrease in  $M_r$  of SNAP-25 from 23.5 to 20.5 K, shown in Fig. 1.

It is noteworthy that SNAP-25 contains other Q-R and R-I peptide bonds, but these peptide linkages are not hydrolyzed, indicating that BoNT/A and /E action, as well as that of the other BoNT serotypes, is not based solely on the recognition of the peptide bond.

#### 4. DISCUSSION

BoNT/A and BoNT/E are zinc-endopeptidases that specifically recognize SNAP-25 as their nerve terminal substrate and cleave it at single, distinct sites within the carboxyl-terminal region. BoNT/A hydrolyzes the Gln<sup>197</sup>-Arg<sup>198</sup> peptide bond, while BoNT/E cleaves the Arg<sup>180</sup>-Ile<sup>181</sup> linkage. This definition of the mechanism of action of BoNT/A and /E leads to a molecular description of the pathogenesis of botulism caused by these two BoNT serotypes. Moreover, a precise knowledge of their specificity is clearly a prerequisite for an appropriate use in the dissection of the molecular events underlying neuroexocytosis.

Although BoNT/A and /E recognize the same nerve terminal target, they cleave it at two peptide bonds of very different chemical characteristics. BoNT/A recognizes a Gln at the P<sub>1</sub> site and an hydrophobic residue at the P'<sub>1</sub> site, as TeTx, BoNT/B and BoNT/F do [7,10]. In contrast, BoNT/E recognizes a positively charged residue at the P<sub>1</sub> site and a hydrophobic residue at the P'<sub>1</sub> site, as it is the case for BoNT/D [12]. Despite these differences, it is intriguing that BoNT/A and /E both cleave within 20 residues of each other at the SNAP-25 COOH-terminus. In comparison, cleavage of VAMP/synaptobrevin by TeNT, BoNT/B, /D and /F and of syntaxin by BoNT/C (Schiavo et al., in preparation) is more centrally targeted and it is thought to disrupt function by dissociating the cytosolic part from its membrane-anchoring segment [3]. Interestingly, while previous studies have not attributed functional importance to the carboxyl-terminal part of the protein, other regions have been implicated in SNAP-25 function. For example, the potential amphipathic helix-forming amino-terminal domain has been suggested to participate in protein-protein interactions [13] and recent studies have shown that the segment 56-94, which includes the palmitoylated cysteine quartet [15], is subject to alternative splicing that may affect neuronal membrane interactions [23,24]. It is remarkable, therefore, that the removal of only nine carboxyl-terminal residues of SNAP-25 by BoNT/A, is sufficient to impair neuroexocytosis. The present findings suggest that this protein region plays a fundamental role in the recognition

and/or regulation of the multi-protein neuroexocytosis machinery.

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