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13. ABSTRACT *(Maximum 200 Words)*

The neuroparalytic symptoms of human botulism, resulting from inhibition of acetylcholine release by type A botulinum neurotoxin (BoNT/A), are life threatening and last for up to 2 years. Thus, development of a fast and effective treatment for the forces exposed to this threat warrants the highest priority. Initial experiments focussed on developing an avid inhibitor of the BoNT/A protease that cleaves SNAP-25 – a SNARE essential for transmitter release. The inhibitors prepared were toxin-resistant mutants of the full-length substrate that retained ability to mediate exocytosis. This vital advance created a means of overcoming the poisoning by transfecting cultured neuroendocrine cells with these SNAP-25 genes. Importantly, the constructs encoding several non-cleavable SNAP-25s rescued exocytosis in BoNT/A-blocked cells, providing an innovative, efficient and rapid therapy for botulism which can be adopted for humans. Moreover, the observed inability of wild-type SNAP-25 to counteract the toxin's action, even at 3 weeks after intoxication, revealed the amazing longevity of type A protease. Our demonstrations that the toxin A –truncation of SNAP-25 (a) disables this SNARE, (b) produces a product that inhibits exocytosis, by competing with its full-length counterpart for binding to the other SNARE partners, and (c) is replenished slowly in neurons, established conclusively that a successful botulism therapy must provide a long-term supply of SNAP-25 engineered to be non-susceptible to the toxin. For its cholinergic targeting via an enzymically – inactive BoNT mutant, the method originally proposed for preparing and reconstituting the individual chains has been superceeded by single chain technology already developed here (see our new proposal). Thus, in ongoing experiments, this targeting vehicle is being utilised in a successful strategy developed for delivering the 'curative' gene into botulinised neurons via innocuous adenoviral vectors whose expression of the functional, but BoNT/A-resistant, SNAP-25 can be controlled. Having established its proof of principle, our proven method for exclusive targeting to the susceptible cholinergic nerves, and regulated expression therein, will be optimised in the next phase of this exciting programme.

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Professor J. Oliver Dolly 14th March '01

PI - Signature Date

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INTRODUCTION

As a prelude to our prime goal of developing an effective therapy for poisoning with botulinum neurotoxin type A (BoNT/A), the contributions of amino acids at the P1, P′1 or P′2 positions in SNAP-25 to its interaction with the active site in the light chain (LC) of BoNT/A have been assessed by site-directed mutagenesis. Wild-type and mutated SNAP-25 were expressed in *E. coli* as glutathionine-S-transferase (GST)-linked proteins and the proteolytic susceptibilities of the resultant mutants to BoNT/A were determined, using developed ELISA methods. Whilst some of these mutants are similarly resistant to the LC protease as peptides previously prepared by Schmidt & Bostian (1997) [where changes were made to a C-terminal 17-mer (187-203) of SNAP-25], attention was focussed here on variants of the full-length substrate because of the demonstration herein of it being cleaved much more efficiently; additional mutations were also investigated. Relative to the smaller synthetic peptides, the much faster cleavage rates by BoNT/A of the full-sized substrate may be attributable to the presence of an important upstream S4-motif (Rossetto *et al.*, 1994; Washbourne *et al.*, 1997). In addition to these fundamental enzymological studies, the most important goal of this research was to produce SNAP-25 gene constructs encoding variants highly-resistant to proteolysis by BoNT/A which should retain ability to support synaptic vesicle docking and fusion when expressed in neurons; these could then be used to examine the molecular basis for the blockade of exocytosis persisting for so many weeks. Ideally, SNAP-25 proteins with single-, double- or triple-point mutations at positions P2 through to P′2 could rescue neurotransmitter release in BoNT/A-paralysed nerves, if successful methods of targeted-delivery were developed.

Whilst the mechanism of BoNT-induced inhibition of neurotransmitter release has received a lot of attention, the molecular processes involved in recovery from poisoning, which can take several months depending on the serotype used, remains poorly understood. In humans, the improvement from neuromuscular paralysis, 49 days post-injection with either BoNT/A- or B is ~ 10% and ~70%, respectively (Sloop *et al.*, 1997). Recently, the physiological basis for this eventual resumption of neurotransmission in poisoned motor-end-plates has been revealed by elegant *in vivo* imaging of synaptic function in the same identified poisoned nerve endings of living mice (de Paiva *et al.*, 1999). These studies proved that full recovery relies on return of synaptic function to the originally-poisoned nerve terminals, though extra-junctional synapses created by newly-formed nerve sprouts underlie the first phase of resumption of neuromuscular transmission. However, it is still unclear if and how the turnover of SNAP-25 and the BoNT/A-truncated form affect the recovery process.

Overview of our Project and Advances.

In order to understand the molecular basis of this protracted intoxication, which seems to vary with the serotype used, a pioneering study funded by USAMRDC was undertaken in isolated adreno-chromaffin

cells (O'Sullivan, *et al.,* 1999). Evoked exocytosis from this convenient cell model shares many characteristics with synaptic vesicle-mediated transmitter release in neurons; in particular, SNAP-25, synaptobrevin (Sbr), and syntaxin1 are present and have all been shown to be essential for large dense-core vesicle (LDCV) exocytosis [for review see (Morgan and Burgoyne, 1997)]. Further, BoNT/A- and /B- induced blockade of exocytosis in chromaffin cells has been shown to be due to cleavage of SNAP-25, synataxin and Sbr/Cbr, respectively (Lawrence *et al.*, 1996; Foran *et al.*, 1996). Finally, previous work on this system had suggested that inhibition of catecholamine release by BoNT/A or TeTx persists for prolonged periods (Bartels *et al.*, 1994). Despite these encouraging features, the validity of using chromaffin cells for studying recovery of release had to be assessed in relation to data reported for the neuromuscular junction (NMJ). In particular, the different profiles of recovery for BoNT/A and B found in humans (Sloop *et al.*, 1997) needed to be determined for chromaffin cells. This would also allow the turnover of the respective BoNT-truncated SNAREs to be directly assessed, a question that cannot be easily answered at the motor nerve terminals. Furthermore, it was hoped to rescue release in BoNT/A-poisoned chromaffin cells through the introduction of vectors-expressing wild-type SNAP-25. The failure to achieve this feat not only provided proof for the continued activity of the toxin for more than 3 weeks but also implied that the SNAP-25 needed to be mutated in such a way that it was profoundly resistant to BoNT/A LC protease, without affecting its ability to support exocytosis. The successful production of BoNT/A-resistant SNAP-25s that are capable of rescuing release establishes that normal release can be restored at any point after BoNT/A poisoning and that these residues are redundant for exocytosis (O'Sullivan *et al.*, 1999).

In advance of adapting our proven strategy for rescue from BoNT/A-poisoning of chromaffin cells to the neuromuscular synapse, we have further characterised the nature of the toxins' blockade of transmitter release using closely related and amenable cerebellar neurons. Following intoxication with type A toxin, extensive inhibition was observed coincident with near-complete cleavage of SNAP-25. Importantly, no significant recovery from this blockade of transmitter release or reappearance of intact SNAP-25 could be detected 31 days after initial exposure to toxin. But detailed studies involving [^{35}S]-methionine labelling and immuno-precipitation of SNAP-25 from neurons poisoned 3 weeks previously, revealed that the LC protease also persists in neurons for long periods (Foran *et al.*, 2001); thus, a useful therapy for botulism must provide a long-term supply of functional SNAP-25 that is not susceptible to the toxin. For this purpose, we recently established recombinant adenoviral methodologies which are known to very efficiently transfect neurons in culture and *in situ* for prolonged periods (see Results). Having created a replication-deficient green fluorescent protein expressing adenovirus, and demonstrated its ability to transduce peripheral neurons in culture, work is currently proceeding to generate BoNT/A-resistant SNAP-25-expressing adenovirus for the rescue of neuroexocytosis in neurons poisoned with BoNT/A. Moreover, a dual strategy will be adopted in the future for treating botulism entailing the arrest of intoxication with inhibitors (being prepared

successfully by Dr. J. Schmidt at USAMRIID; Schmidt *et al.*, 1998) whilst replenshing the toxic SNAP-25_A with full-length BoNT-resistant SNAP-25. Both treatments will involve targeting of the viral constructs, and the small inhibitors, to cholinergic nerves using atoxic BoNT/E available in high yields from our innovative single-chain expression system.

BODY

MATERIALS AND METHODS

Materials.

Highly purified BoNT/A, /B and /E were supplied by Drs Gary Lawrence (Imperial College, London), Eric Johnson and Bibhuti DasGupta (Madison, Wisconsin). Antibody preparations reactive with C-terminus of SNAP-25 were raised against a synthetic peptide encompassing residues 195-206 and affinity-purified on the immobilised immunogen. Urografin (Schering Healthcare, G.D.R.), digitonin (Novabiochem, U.K.), radio-immunoassay (RIA) kit for human growth hormone (hGH) (Nichols Institute, San Capistrano, CA, USA), Quick-Change™ (Stratagene, Netherlands), calcium phosphate reagents, cell culture reagents (GIBCO-BRL, U.K.), Superfect™ (Qiagen, U.K.), pGEX-2T (Amersham Pharmacia, U.K.) and pcDNA1.1 (Invitrogen, The Netherlands) were purchased. Mouse cDNA clones (GST-SNAP-25) containing the entire coding region of SNAP-25b (206 residues) (Zhou *et al.*, 1995) and GST-SNAP-25_A, a BoNT/A-truncated SNAP-25 (residues 1-197) were prepared in this laboratory. ELISA plates (Probind or Microtest III) were purchased from Becton Dickenson (Crawley, Oxford). Fraction V bovine serum albumin (BSA) and Immobilon-P membrane were from ICN Flow Ltd. and Millipore. Enhanced chemilumenescent substrates were purchased from Amersham International (Amersham, U.K.). All the other reagents used were of the highest purity available and supplied by Sigma Chem. Co. Ltd. (Poole, Dorset) including the Tris salt of *p*-nitrophenyl-phosphate (pNPP), anti-species specific antibodies conjugated to horseradish peroxidase or alkaline phosphatase. The suppliers of other specialised products are detailed in the Methods.

Expression of GST-SNAP-25 in *E. coli.*

GST-SNAP-25 and GST-SNAP-25_A were expressed using methods described by Smith and Johnson (1988). Bacteria containing the pGEX-2T plasmid incorporating the appropriate SNAP-25 gene were grown overnight at 37°C with vigorous shaking in 5 ml of Lauri-Bertani (LB)-medium containing 100 μg/ml ampicillin and diluted 200-fold into 1 L of the same medium. This mixture was incubated at 37 °C with vigorous agitation until an optical density of 0.6 units at 600 nm was reached; at this stage, isopropyl-β-D-thiogalactopyranoside (IPGT) was added to 0.4 mM concentration and the incubation continued for 4 h. The bacteria were harvested by pelleting at 4,000 g (max) for 10 min; this and all

subsequent procedures were performed at 4°C. The resultant bacterial pellet was resuspended in 10 ml Tris-buffered-saline [TBS; consisting of 25 mM Tris.HCl, pH 7.5 containing 0.15 M NaCl) supplemented with 2 mM EDTA, 0.5 mM PMSF, 0.5 mM benzamidine, 1 mM DTT, 5% glycerol, 1 mg/ml lysozyme and one of the following detergents: 1% (w/v) Lubrol-PX (also known as Thesit) or Triton X-100. Bacteria were then lysed by brief sonication whilst on ice; insoluble material was removed by ultracentrifugation at 200,000 g (max) for 30 min. The resultant supernatant was retained and subjected to affinity chromatography.

Purification of GST-SNAP-25 from bacterial lysates by affinity chromatography.

The resultant high-speed supernatant was added to 2 ml of glutathione-Sepharose and the mixture incubated batch-wise by gentle rotary shaking at 4°C for 1 h. The mixture was added to a 10 ml column and the resultant breakthrough put through at least 3 times to maximise binding. Next, the resin was washed using 10 x 5 ml aliquots of 2 x strength TBS buffer to remove proteins interacting non-specifically. Finally, the column was equilibrated with 5 ml of TBS, prior to bio-specific elution of the fusion proteins with 10 x 1 ml aliquots of TBS buffer (pH 7.5) containing 10 mM glutathione and 10% (v/v) glycerol. The protein content was determined using a colourimetric method (Bradford, 1976) and the pooled material frozen at -60°C as aliquots. When necessary, purified recombinant GST-fusion proteins was incubated with thrombin (purified from human plasma; activity ~ 200 units/mg protein; Sigma Chem. Co. Ltd.) in TBS buffer, pH 7.5 for 2 h at 37°C at 1:100 (w/w) enzyme:substrate ratio to cleave the susceptible bond located between GST and SNAP-25.

Coating GST-SNAP-25 onto ELISA plates.

Polystyrene 96-well plates were incubated with GST-SNAP-25 (50 µl of 2 µg/ml per well) in a modified KGEP buffer [consisting of: 20 mM piperazine-N-N`-bis 2-ethanesulphonic acid (PIPES).NaOH, pH 7.0, 140 mM potassium glutamate, 5 mM $MgCl_2$, 0.5mM EGTA, 50 µM $ZnCl_2$ and 0.02% (w/v) NaN_3] for a minimum of 1 h at 23°C. Unbound substrate was removed by brief rinsing with 2 x 0.15 ml aliquots of TBS buffer. The wells were blocked for a minimum of 1 h at 23°C, or overnight at 4°C, by incubation with 0.15 ml aliquots of TBS containing 2% (w/v) BSA (fraction V) and 0.02% (w/v) NaN_3.

Synthesis and purification of SNAP-25 peptides.

Peptides were synthesised commercially (Genosys or Biosynthesis Inc) using F-moc methods. All peptides were purified on reverse phase-HPLC and judged to be at least 90% pure; mass spectrometry was used to confirm their appropriate molecular weights.

Covalent linkage of synthetic peptide immunogens to carrier proteins.

For the purpose of generating anti-sera, synthetic peptides CTRIDEANQ or CRIDEANQ covalently-linked to key-hole limpet heamacyanin (KLH) for improved immunogenicity. The N-terminal cysteine which had been added allowed coupling of the peptides to maleimide-activated (i.e. thiol-reactive) KLH (Pierce and Warriner). To ensure efficient coupling of the thiol-containing peptides to KLH or BSA, the thiol contents were monitored (Ellman, 1959).

Immunisation of animals with peptide-KLH conjugates.

Guinea-pigs were immunised by injection at 4 sites (2 intramuscularly and 2 sub-cutaneously for each animal) with 0.2-0.4mg of peptide conjugates mixed with an equal volume of Freunds adjuvant (vortexed vigorously for 2-4 min until a stable emulsion formed). Freunds complete adjuvant was used for the first injection (day 1) and subsequent booster injections performed on days 14, 28 and 42 employed Freunds incomplete adjuvant. Animals were sacrificed and bleed out on or after day 54. Blood was incubated at 37°C for 2 hr to accelerate clotting and the serum was recovered as a supernatant following centrifugation at 15, 000g (max) for 30 min at 4°C.

Characterization of the reactivities of IgG preparations towards SNAP-25$_A$ or the intact protein using dot-blotting.

Each immune serum, and the final purified IgGs, were tested by dot blotting to ensure that each labelled the appropriate antigen. Varying amounts of GST SNAP-25 or GST SNAP-25$_A$ were deposited as 2 µl spots (in the TBS buffer containing 100 µg/ml Thesit detergent) onto wetted Immobilon-P membranes (either: 0, 0.3, 1, 3 and 10 ng/spot). After being allowed to dry, the membranes were washed briefly with TBS containing 0.1% (v/v) Tween 20 and blocked in TBS containing 5% (w/v) skimmed milk powder prior to addition of primary Ig. Antibody binding was detected using anti-species specific Ig conjugated with alkaline phosphatase and visualised by means of the colourimetric substrates 5-bromo-4-chloro-3 indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

Covalent-coupling of thiol-containing SNAP-25 peptides to iodoacetyl-activated Sepharose 4B.

SNAP-25 immunogenic peptides were linked to resins for use in the affinity-purification of specific antibodies. Iodoacetyl-Sepharose 4B was prepared using the method of Hermanson *et al.* (1992), in preference to the more popular maleimide activation procedure; this avoided co-isolation of Ig generated against the highly immunogenic maleamide cross-linker used for peptide carrier conjugation.

Isolation of specific Ig preparations by affinity chromatography.

In order to recover the Ig species from serum with exclusive selectivity for SNAP-25_A, reactivity to the full-length molecule was removed from the crude immune sera by pre-incubation with Sepharose resin conjugated with the SNAP-25 peptide corresponding to residues 181-206. In all cases, 2 ml of serum (mixed with 2 ml of TBS) were incubated batchwise with 1 ml of resin coupled with 0.5 mg of peptide in a end-over shaker for 4 h at 23°C, followed by addition of the mixture to a column and repeated passage of the sample (2-3 times) at a flow rate of 0.5-1 ml min^{-1}. Affinity isolation of the appropriate antibodies from the latter breakthrough was achieved by passing ~ 5mL of 'depleted' immune serum through a column containing ~ 2 ml of CTRIDEANQ peptide covalently-coupled to Sepharose, 3 or 4 times over 20-30 min. After collection of the breakthrough, the column was washed with 5 x 3ml aliquots of 0.1 M Tris.HCl pH 7.5 buffer containing 0.75M NaCl to remove proteins adsorbed non-specifically to the resin. Finally, the column was equilibrated with 5ml of TBS buffer and bound anti-SNAP-25 peptide Ig eluted using 10 x 1 ml aliquots of 0.1 M glycine.HCl (pH 2.6) buffer containing 0.2 M NaCl which were collected separately into 0.15 ml of a pH neutralising buffer (1 M Tris.HCl pH 8.5). Protein concentrations were calculated from absorbancies at 280 nm (1 mg Ig/ml ≡ 1.35 Units cm^{-1}) and homogeneity was confirmed by SDS-PAGE.

Assessment of the susceptibilities of wild-type and mutant SNAP-25 to cleavage by BoNT/A using ELISA.

Purified full-length GST-SNAP-25 was coated on 96-well plates, rinsed, blocked and exposed to various concentrations of DTT-reduced BoNT/A at 37 °C for the periods specified in the Figure legends. The wells were then aspirated, washed and probed with anti-SNAP-25(C-terminus)-IgG (Lawrence, *et al.,* 1996) or anti-SNAP-25_A specific-pAb. Unbound IgGs were removed by rapid washing and the primary IgGs bound detected indirectly, using anti-species-specific IgGs-covalently conjugated to alkaline phosphatase, and visualised by colour development upon addition of p-nitrophenyl phosphate substrate. The A405nm values recorded from toxin-treated wells were expressed as percentages of toxin-free control values and plotted against the toxin concentration used. Standard

curves relating amounts of intact SNAP-25 remaining in wells were constructed, using defined mixtures of full-length or BoNT/A-truncated GST-SNAP-25. The A405nm readings recorded were expressed as percentages of that recorded for the 100% intact protein sample and plotted against the amounts of intact GST-SNAP-25 coated initially. Generally, between 60 and 70 % of the maximal A405nm signal recorded represented 50 % intact SNAP-25 in wells (depending on the primary Ig used).

Alternatively, a modified ELISA was employed to determine the initial rates of cleavage by BoNT/A of GST-SNAP-25 at a range of substrate concentrations. GST-SNAP-25 concentrations (0.118 - 15 μM) in KGEP buffer pH7.2 (buffer detailed in Lawrence *et al.*, 1996) containing 10 μg/ml BSA and 50 μM $ZnCl_2$ were incubated, in the absence or presence of 0.05 nM reduced BoNT/A, for several time periods chosen to yield less than 15 % cleavage. Reactions were quenched by adjusting pH to 10 and by addition of 20 mM EDTA using a suitable buffer. Next, each GST-SNAP-25 sample was diluted to the same concentration (4 μg/ml) and coated onto multiple wells of a 96-well plate. In addition, mixtures of full-length and BoNT/A-truncated SNAP-25 ($SNAP\text{-}25_A$) were also coated onto wells in order to generate a standard curve (see below). After incubation of wells with the Ig that only recognizes $SNAP\text{-}25_A$ (i.e. anti-$SNAP\text{-}25_A$-pAb), unbound IgG was removed by washing and bound Ig detected using horseradish peroxidase-conjugated anti-species Ig. After washing, bound secondary Ig was visualised using a colour reaction that was measured at 405 nm. Standard curves relating % of $SNAP\text{-}25_A$ of total SNAP-25 protein coated were plotted against the $A_{405\,nm}$ values recorded per well. A linear correlation was observed between increases in absorbance and the % of $SNAP\text{-}25_A$ coated; thus, the % $SNAP\text{-}25_A$ present in the wells of unknown samples could be extrapolated and used to calculate initial rates.

Primer design and synthesis.

Oligonucleotide primers, utilised for the mutagenesis of SNAP-25, were designed using the PrimerSelect program (Lasergene, Madison, USA) in order to optimise the compatibilities of their melting temperatures. Primers were synthesised by MWG Biotech Ltd. (Milton Keynes, UK.).

PCR amplification and multiplication reactions.

PCR reactions were performed using a RoboCycler Gradient PCR machine (Stratagene), which allows multiple annealing conditions to be evaluated during the same PCR cycle. All PCR amplification reactions were performed using PCR core system II (Promega) which enabled optimisation of magnesium concentrations critical to the activity of *Taq* DNA polymerase. PCR reaction mixtures were

overlaid with 30 µl of sterile paraffin wax to prevent volume fluctuations during thermo-cycling, due to evaporation and condensation.

Two-step PCR mutagenesis.

Site-specific point mutations of the SNAP-25 gene (for NQR**L**, NA**A**A, NA**K**A, NW**W**A, **QAK**A and NA**K**L; mutations in bold) were introduced using two independent PCR reactions (reviewed in Higuchi, 1990). The first PCR involved the introduction of the desired mutation using the isolated wild-type SNAP-25 gene as a template plus two primers. The forward primer was complementary to the sense start sequence of the wild-type gene with the reverse primer being complementary to the antisense sequence of the SNAP-25 cleavage site but incorporating the appropriate mismatched bases. The amplified PCR fragment was shortened at the 3' end by ≈ 27 bases, depending on the primer used. A second PCR was subsequently performed to replace the missing bases. This utilised the same forward complementary primer as before, in conjunction with a new reverse antisense primer entirely complementary to the wild-type sequence at the 3' end and also partially complementary to the antisense sequence of the truncated PCR product. Although this two-step PCR method is more labour intensive than the one-step procedure described next, its main advantage is that two smaller independent primers could be used instead of much longer oligonucleotides needed for a single PCR reaction; this saves cost and avoids problems encountered with the synthesis and isolation of long primers (i.e. n-1 products), plus the increased likelihood of secondary structure formation (i.e. hairpin loops etc.).

One-step PCR mutagenesis.

This method (reviewed in Higuchi, 1990) was used to produce the following variants: residues 1-199; 1-200; 1-201; 1-202; 1-203 (SNAP-25_{FEW}); 1-198 (SNAP-25_{C1}); 117-206 (SNAP-$25_{117\text{-}206}$, N-terminal deletion); 143-206 (SNAP-$25_{143\text{-}206}$, N-terminal deletion); 1-206 +18 (SNAP-25_{ADD}, addition of 18 C-terminal residues). The C-terminal deletion mutants were produced by incorporating a stop codon in the reverse primer sequence at the desired site. The N-terminal deleted forms, SNAP-$25_{117\text{-}206}$ and SNAP-$25_{143\text{-}206}$, were produced by using forward primers complementary to the desired regions of deletion of the wild-type template sequence. The addition mutant, termed SNAP-25_{ADD}, was obtained by incorporating a complementary overhang into the pGET-2T vector on the reverse primer at 3' end; this introduced additional codons followed by a stop codon downstream of the SNAP-25 and vector sequence. All forward primer sequences also contained the *Bam*H1 restriction site whilst the reverse primers included the *Eco*R1 site to enable uni-directional ligation into pGEX-2T vector.

Ligation of mutant SNAP-25 genes produced by one or two-step mutagenesis PCR methods into bacterial expression vectors.

The altered SNAP-25 constructs were analysed by agarose gel electrophoresis and fragments of the appropriate size isolated from low-melting point gels. PCR-generated wild-type and mutant SNAP-25 genes were treated with *Eco*R1 and *Bam*H1 to generate the appropriate overhanging ends necessary for directional ligation into the pGEX-2T vector (performed using T4 DNA ligase), which were then transformed into bacteria by electroporation.

Dpn-1 nuclease-quick change PCR mutagenesis.

All the remaining site-specific mutations of the SNAP-25 gene were made, whilst it was still within the double-stranded pGEX-2T plasmid, using PCR-mediated plasmid multiplication (detailed in Bergsied *et al.*, 1991). Sense and antisense oligonucleotide primers complementary to opposing strands at the modification site (i.e. incorporation of appropriate mismatches) were designed to have relatively high melting temperatures to ensure that the primer pairs remained annealed to the plasmid template during thermo-cycling extensions. The PCR multiplication was performed by temperature cycling with *Pfu* DNA polymerase (an enzyme exhibiting a very low error rate), using the pGEX-2T plasmid containing the SNAP-25 gene as the parental template. Following PCR, the mixture containing the newly synthesised nicked DNA plasmids was subjected to digestion using *Dpn*-1 endonuclease which only degrades the methylated, non-mutated parental template (target sequence: 5'-G^{Meth}ATC-3'). The remaining mutated double-stranded plasmid DNA was transformed into the BL21 bacterial strain by electroporation and selected on agar plates containing ampicillin. Sequencing was carried out on an ABI automated sequencer to confirm that the sequences had been correctly mutagenised.

Culture of cells and intoxication with BoNT/A or /B.

Bovine chromaffin cells were prepared from adrenal glands and maintained as primary cultures as described previously (Lawrence *et al.*, 1994). Cells required for transfection were further enriched (to remove any contaminating fibroblasts) using Urografin density-gradient centrifugation, as detailed by Wilson (1987). Within 2-3 days after preparation, cells were incubated with low ionic strength medium (LISM) at 37°C for 24 h in the absence or presence of 6.6 nM BoNT/A or 66 nM BoNT/B and maintained at 37°C for up to 56 days, by weekly replacement of medium (O'Sullivan *et al.*, 1999).

Stimulation and quantification of catecholamine secretion from intact chromaffin cells.

Immediately before measuring release, cells pre-treated with or without BoNT/A or /B were briefly washed with Locke's solution (Wilson, 1987) and then incubated in quadruplicate at 22°C in this buffer, with and without the inclusion of 2 mM Ba^{2+}. After 15 min, aliquots of the medium bathing the cells were removed and assayed for catecholamine content, using a fluorometric procedure (Lawrence *et al.*, 1996). Values for basal release of catecholamine were subtracted from the respective evoked components and expressed as a percentage of the total content.

Expression of wild-type or mutant SNAP-25 in cells: sole monitoring of transfected cells using a hGH-based reporter system.

Wild-type and mutated SNAP-25 genes (listed in Figure 8) generated by PCR-based mutagenesis techniques (see 1st Annual Report) were introduced into the mammalian expression vector pcDNA1.1/Amp from pGEX-2T, using the *Bam*H1 and *Eco*R1 restriction sites. Control or 6.6 nM BoNT/A-treated chromaffin cells were transfected with the hGH construct together with that of a control - chloramphenicol acetyl transferase (pcDNA1.1/Amp-CAT) - or the appropriate SNAP-25 gene, using the calcium phosphate precipitation method (Holz *et al.*, 1995). Four to six days after transfection, hGH secretion from intact chromaffin cells was stimulated and quantified, as described for catecholamine release except for the use of a RIA. Some transfected cells were permeabilised with 20 μM digitonin in a permeabilisation buffer and exposed for 15 min to reduced BoNT/E. Evoked release was assessed over a subsequent 15 min by the addition of 20 μM free Ca^{2+} (Lawrence *et al.*, 1997). Aliquots were removed and assayed for hGH content; Ca^{2+}-evoked hGH secretion was calculated as described above.

Assessment of the proteolytic activities of BoNT/A or /B in cell cultures.

CHO cells, which lack SNAP-25, were plated at 50-70% confluency on a 6 well plate. After treatment with BoNT/A in LISM as detailed for chromaffin cells, CHO cells were transfected with pcDNA1.1/Amp-SNAP-25s using Superfect™. Membranes were isolated from the latter, and chromaffin cell samples, as previously described (Lawrence *et al.*, 1996; Foran *et al.*, 1996), subjected to immunoblotting with IgGs including anti-SNAP-25 (recombinant)-IgG (an antibody raised against full-length recombinant GST-SNAP-25), anti-$SNAP\text{-}25_A$-pAb (an antiserum solely reactive with $SNAP\text{-}25_A$), anti-SNAP-25 (C-terminus)-IgG (generated against the last 12 residues of SNAP-25) and anti-Sbr/Cbr-IgG (Lawrence *et al.*, 1996). Bound antibodies were detected indirectly using anti-

species-specific IgGs-covalently conjugated to horseradish peroxidase and visualised by the enhanced chemiluminesence (ECL)-detection system.

Preparation and maintenance of cerebellar granule neurons and assay of glutamate release.

These cells were dissociated from the cerebella of 7-8 day old rats, using the method of Camray-Deakin (Cambray-Deakin, 1995). Neurons were suspended at 1-2 x 10^6/mL in medium consisting of 3 parts Basal Eagle's Medium and 1 part of [(mM): 40 HEPES-NaOH pH 7.3, 78.4 KCl, 37.6 D-glucose, 2.8 $CaCl_2$, 1.6 $MgSO_4$ and 1.0 NaH_2PO_4)], as well as 1 x N2 supplement, 1 mM L-glutamine, 60 units/mL penicillin, 60 μg/mL streptomycin and 5% (v/v) dialysed horse serum. An aliquot of this cell suspension was added to each 22 mm diameter poly-D-lysine coated well, cytosine-β-D-arabinofuranoside (40 μM) was added after 20-24 h in 5% (v/v) CO_2 culture and the neurons maintained by weekly replacement of the same medium. Neurons cultured for at least 10 days were washed 4 times with O_2 –gassed Krebs-Ringer HEPES ([KRH], mM: 20 HEPES NaOH pH 7.4, NaCl 128, KCl 5, NaH_2PO_4 1, $CaCl_2$ 1.4 , $MgSO_4$ 1.2 , D-glucose 10 and BSA 0.05 mg/mL), and 0.5 ml of the latter buffer containing 0.25 μCi/mL [^{14}C]-glutamine (i.e. a glutamate precursor (Gallo *et al.*, 1982) was added. All steps were performed at 37°C. After a 45 min labeling period, the neurons were washed 4 times as before, and incubated for 5 min at 37°C in KRH containing either 1.4 mM Ca^{2+} or 0.5 mM EGTA (i.e. to assess Ca^{2+}-independent release); aliquots were removed and retained for measurement of [^{14}C]-glutamate content by ion-exchange HPLC analysis and scintillation counting. A modified KRH buffer containing 50 mM KCl (with a reduced NaCl content of 83 mM to maintain osmolarity) and either 1.4 Ca^{2+} or 0.5 mM EGTA was added for a 5 min stimulation period. Finally, neurons were solubilised with 20 mM EGTA.NaOH pH 7.5 containing 1% (w/v) SDS. The amounts of [^{14}C]-glutamate in basal and stimulated samples were expressed as % of the total cell content. The amounts of KCl-induced [^{14}C]-glutamate released into EGTA-containing buffer were subtracted from the values recorded from Ca^{2+}-containing samples in order to calculate the Ca^{2+}-dependent component of evoked release.

Subcloning of SNAP 25 genes and green fluorescent proteins (GFP) into recombinant adenovirus.

The Adeno-X Tet-Off system (Clontech; shown diagrammatically in Fig. 21) utilises a pTRE shuttle vector that allows rapid insertion of the gene of interest into the adenoviral DNA (relative to the earlier very time-consuming homologous recombination). Thus, the genes encoding SNAP-25 variants (wild-type, Q198T, 1-197, 1-181) or GFP were incorporated into the pTRE shuttle vector and their presence confirmed by restriction digest analysis. The cassette encompassing the CMV Tet response element

(TRE) promoter and gene was excised from the latter (Fig. 21) and ligated into pre-cut Adeno-X viral DNA; non-recombinants were excluded by the retention of a Swa1 site (Fig. 21). After transforming *E. coli*, the recombinant viral vectors were selected on ampicillin plates, amplified and plasmid DNA purified. Prior to transfecting HEK 293 cells (see below), the DNA had to be linearised using Pac 1. Full details of the methods outlined above are available at the Clonetech web site: www.clonetech.com.

Transfection of HEK-293 cells with GFP-expressing adeno-X viral DNA in order to generate recombinant adenovirus.

For safety, the E1 and E3 elements essential for viral replication have been deleted and, thus, the Adeno-X virus can only replicate in the HEK-293 cell line engineered to provide that protein. Twenty four hours before transfections, 35 mm tissue culture wells were inoculated with ~ 2 x 10^5 HEK-293 cells and maintained at 37°C, 5% (v/v) CO_2 in medium consisting of minimal essential medium (alpha-modification) supplemented with 10 mM HEPES pH 7.5, 15 mM D-glucose, 4 mM L-glutamine, 100 units/mL penicillin G, 100 μg/mL streptomycin and 10 % (v/v) Tet-system approved fetal bovine serum (i.e. tetracycline-free; Clonetech). Adeoviral DNA containing the GFP gene was transfected into HEK-293 cells using GeneJammer™ (Stratagene) reagent using the protocols detailed at the manufacturers website: www.stratagene.com. Four passages of the cells was required before most of the cells had detached, indicative of viral production. The resultant cells were lysed hypotonically and sheared (to release virus) and the resultant supernatant (~ 10,000g x 10 min) was combined with the centrifuged culture medium (also containing virus). Viral titre was determined using a kill assay (detailed in O'Carroll *et al.*, 2000). High-titer stocks of GFP-expressing TRE and Tet-off regulatory viruses were prepared by infecting HEK-293 cells (plated at ~ 70 % confluency) with 2-5 plaque forming units (p.f.u.)/cell, using the low titer samples generated above. After 2-3 days in culture, most of the cells detached and were collected by low speed centrifugation (2,000 g max x 5min). Although the resultant cleared culture medium contains up to half (variable) of the total adenovirus generated, the fetal calf serum present which kills cultured neurons precludes its use. Instead, useful virus was isolated from the infected cells by lysis. These cells were washed once with HEPES buffered saline pH 7.5, recollected as before, suspended in 20 mM HEPES pH7.5 and stored on ice for 20 min, prior to final shearing by four up-down passes through a blunt-ended 26 gauge tip. The lysate was adjusted to 150 mM NaCl (using a 10-fold concentrated stock) and the virus-containing supernatant resulting from a 15,000 g spin for 10 min was stored in aliquots at −80°C. Prior to use, viral titers were determined as outlined above. Generally, ~ 10^9 p.f.u. of adenovirus were produced per 175 cm^2 flask. All viral

production and manipulations were performed using Biosafety Level 2 containment and working practices.

Adenoviral infection of neurons in culture and detection of expression of β-galactosidase (β-gal) or GFP visualisable markers.

In addition to replication of the adenovirus being restricted, another level of safety is in-built by the necessity to add a second virus, termed the Adeno-X Tet-Off regulatory component. The latter contains the gene encoding the Tet transactivator protein, which activates the CMV TRE promoter in the Adeno-X TRE virus and allows expression of the gene product. This system enables tetracycline-induced inhibition of expression of the gene of interest. Target neurons were exposed to the amounts of viruses specified in the legends to figures 20 and 22 using normal conditions for 2-3 days, prior to analysis for marker protein expression. GFP expression was visualised in living neurons by fluorescent microscopy and the presence of β-gal activity observed using a chromogenic substrate precipitation assay. To detect β-gal, neurons were rinsed thrice with PBS and fixed with 0.5 % (w/v) glutaraldehyde in PBS for 5 min. Fixed cells were rinsed thrice with PBS and incubated in staining solution [5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ and 0.25 mg/mL 5-bromo-4-chloro-3-indolyl-β D-galactopyranoside in PBS], until the nuclei of infected cells stained blue which were observed by microscopy.

RESULTS AND DISCUSSION

Expression and purification of recombinant GST-linked SNAP-25s.

The entire coding region of SNAP-25 (1-206) or that for the BoNT/A-truncated N-terminal moiety (1-197) were inserted into pGEX-2T vector for expression in *E. coli*. In each product, GST was attached to the N-terminus separated by a linker region containing a thrombin-sensitive site. Cytoplasmic extracts from the cultures were passed through a column of glutathione Sepharose; SDS-PAGE analysis revealed that the breakthrough had a lower content of a ~ 48 kDa protein than the sample loaded (Figure 1 A, C), indicating appreciable retention of the GST-SNAP-25 proteins by the affinity resin. Following washing to remove non-specifically-interacting proteins, GST-linked fusions were eluted biospecifically using glutathione (Figure 1A, C). The sizes of the eluted proteins were consistent with GST (~ 23 kDa) being linked to SNAP-25 (~25 kDa). Up to 5 mg of GST-SNAP-25 protein was isolated from a 1 L culture. The identities of GST SNAP-25s were confirmed by their reactivity on immunoblots with different Ig preparations reactive with the 12 C-terminal residues of SNAP-25,

termed anti-SNAP-25 (C-terminus) for detecting the full-length molecule (Figure 1 B) or GST-SNAP-25_A - an antiserum uniquely reactive with the BoNT/A truncated N-terminal fragment (termed anti-SNAP-25_A; Figure 1D; supplied by Dr. T.A. Ekong). Whilst affinity-isolated GST-SNAP-25 was reasonably pure, being > 80% (Figure 1A) as assessed by densitometric scanning of gels, GST-SNAP-25_A contained a significant proportion of free GST (Figure 1C).

BoNT/A efficiently proteolyses GST-SNAP-25.

To ensure that bacterially-expressed GST-SNAP-25 is a good substrate for BoNT/A, *in vitro* cleavage experiments were performed. GST-SNAP-25 was incubated in the absence or presence of either 2 or 5 nM of pre-reduced BoNT/A for 30 min at 37°C. Immunoblotting performed using either anti-SNAP-25 (C-terminus) -Ig or anti-SNAP-25_A revealed that GST-SNAP-25 was efficiently cleaved by BoNT/A, as judged by disappearance or appearance of reactive signals, respectively (Figure 2).

Production of Igs solely reactive with SNAP-25_A.

In order to replenish the limited sample of anti-SNAP-25_A serum supplied by a colleague, attempts were made to use published methods (Hallis *et al.*, 1996; Ekong *et al.*, 1997) to generate sufficient quantities of antiserum for use in the future assessment of BoNT/A endopeptidase activity. The peptides CRIDEANQ or CTRIDEANQ, corresponding to the N-terminal sequence immediately upstream of the BoNT/A cleavage site in SNAP-25, were covalently-coupled to maleimide-activated KLH (through the additional N-terminal cysteine incorporated) to improve immunogenicity. Antisera were generated in guinea-pigs (3 per antigen) using an immunisation protocol detailed in Methods. However, the resultant antisera were found to react with both GST-SNAP-25_A and the intact protein, using the dot-blotting procedure outlined. The sample of anti-SNAP-25_A serum provided by Dr. Ekong did react appropriately detecting GST-SNAP-25_A with high avidity and exhibiting no cross-reactivity with the full-length molecule, confirming the suitability of this method of assessment.

In order to recover the appropriate Ig reactivity from our antisera, attempts were made to deplete reactivity towards the full-length molecule by absorption onto a peptide which encompasses the C-terminus of SNAP-25 that had been covalently coupled to Sepharose. Antisera from all guinea-pigs were incubated with excess SNAP-25-(181-206)-Sepharose followed by repeated passage through the column and retention of the final breakthrough. Finally, the anti-SNAP-25_A-specific-Ig was isolated from the latter 'depeleted' sera by adsorption onto CTRIDEANQ-Sepharose and elution from the resin using a low pH buffer. Unfortunately, all of the SNAP-25-(181-206) pre-depleted Igs generated using the CTRIDEANQ-KLH antigen reacted with SNAP-25_A only marginally better than the intact

molecule (data not shown), excluding their future use. Fortunately, several of the SNAP-25_{FL}-depleted purified Ig preparations obtained from antisera generated against the CRIDEANQ-KLH antigen displayed high degrees of selectivity for SNAP-25_A over SNAP-25_{FL}; dot-blotting demonstrated that these (from guinea-pig 3 and, especially, guinea-pig 1) exhibited selectivity equal to or exceeding that of anti-SNAP-25_A serum provided by Dr. Ekong (Figure 3). The purified Ig from guinea-pigs 1 and 3 are referred to as anti-SNAP-25_{A1} Ig and SNAP-25_{A2} for simplicity. Conversely, the anti-SNAP-25 (C-terminus)-specific Ig prepared selectively reacted with intact SNAP-25 (Figure 3).

Development and characterization of an ELISA for monitoring the selective proteolysis of recombinant SNAP-25 by BoNT/A.

The appropriate reactivities of the anti-SNAP-25_{A1}, SNAP-25_{A2} and anti-SNAP-25 (C-terminus) Ig preparations demonstrated for either SNAP-25_A or the intact molecule, enabled us to assess GST-SNAP-25 cleavage by BoNT/A using the ELISA method. After coating 96-well polystyrene plates with GST-SNAP-25 and blocking with BSA (see Methods), wells were exposed to increasing concentrations of BoNT/A. Appearance of the SNAP-25 product (termed SNAP-25_A) resulting from BoNT/A proteolysis was detected using anti-SNAP-25_{A1} Ig. The amounts of Ig retained following washing was quantified using anti-species-specific Ig covalently-conjugated with alkaline phosphatase, via a colourimetric reaction (detailed in Methods). As anticipated, the absorbance values increased in response to the quantity of toxin added. In support of the validity of this method, BoNT/A dichain required disulphide-reduction to cause this increased absorbance (i.e. cleavage of SNAP-25 by type A had occurred; Figure 4A). In separate experiments using anti-SNAP-25_{FL} Ig, that recognises the last 12 C-terminal amino acids of SNAP-25, to follow disappearance of intact substrate rather than the product of BoNT/A cleavage, a gradual dimunition of immunoreactivity in response to the increased amounts of toxin was observed (Figure 4B). Encouraged by the success in assaying BoNT/A cleavage by ELISA, we improved this assay for quantitative proteolytic measurements (see next section).

Construction of standard curves relating amounts of SNAP-25_{FL} or SNAP-25_A to the observed absorbance changes allowed quantitation by ELISA of the amount of SNAP-25 cleaved by BoNT/A. To ensure the accuracy of the ELISA for measuring the extents of cleavage of SNAP-25, and to achieve conditions necessary for quantitative measurements, a variety of parameters were assessed. It is essential that primary Ig should be present in excess over the amounts of substrate or cleaved product present in wells so that all sites can be saturated. Similarly, excess enzyme-linked secondary Ig is necessary for complete binding to all primary Ig retained on wells following washing. In addition, the concentration of the colourimetric substrate used to visualise the enzyme-linked secondary antibody

should be sufficient such that for the absorbance values plotted (i.e. up to 2 units used here) result from less than 10% having been utilised (i.e. linear rate of development; see Methods).

To facilitate the future quantitative assessment of the amounts of either the intact substrate or the N-terminal product (SNAP-25_A) present in wells resulting from BoNT/A action, standard curves were constructed. Defined mixtures of GST-linked SNAP-25 or SNAP-25_A (between 0 and 100% of the total protein) were coated onto wells to mimic the effects of exposure to toxin. The primary Ig anti-SNAP-25_{A1} was incubated with wells, detected using enzyme-linked secondary antibodies and developed colourimetrically for the indicated periods (Figure 5A). As expected, A405 nm values increased in proportion to the amounts of SNAP-25_A but not in a perfectly linear manner. However, a good linear relationship was obtained between increased A405 nm and amounts of SNAP-25_A between 0 and 10% of the total protein (Figure 5B). The latter observation is particularly encouraging because this assay will be used to measure rates of BoNT/A action in the future. It is preferable that constant amounts of SNAP-25_A (up to 10% of the total amount of intact substrate) are coated into wells so that an upper-limit is adhered to, below which absorbance values can be used with accuracy. In order to assess the overall relationship between the increase of A405 nm and the proportion of SNAP-25_A on wells, the absorbances recorded after six minutes were expressed as % of the maximum signal measured and plotted against the appropriate amount of GST SNAP-25_A product (Figure 5C); the dotted line indicates where data points would lie if a perfect linear relationship existed. Good correlations were obtained with only minimal divergences (up to ~ 15%) (Figure 5C). The latter plot will be used to derive the actual quantities of SNAP-25_A product in wells compared to the uncleaved substrate, in future experiments.

The quantitation of intact substrate remaining in samples, following BoNT/A-proteolysis, is also important. Therefore, ELISAs were performed to relate the increase of A405 nm signals with the different percentage of SNAP-25 coated, using anti-SNAP-25 (C-terminus) specific-Ig (Figure 5D). A405 nm measurements recorded alter 3 or 6 min developments are plotted, giving similar curves (Figure 5D). To enable visual assessment of the relationship between the increases of A405 nm and the proportion of intact SNAP-25 on wells, the absorbance values recorded after three minutes of development were expressed as % of the maximum value recorded. Although the increments in A405 nm when plotted against amounts of intact SNAP-25 (% total fusion protein) vary by up to ≈ 20% from the perfect linear (indicated by dotted line; Figure 5E), the latter standard curve can nevertheless be used in future experiments to calculate the amounts of intact SNAP-25 remaining following BoNT/A exposure.

BoNT/A proteolyses full-sized SNAP-25 more efficiently than small peptide substrates: co-incubation with S4-motif peptides does not improve protease activity.

In pursuit of a minimal-sized efficient substrate of BoNT/A on which to base the development of inhibitors, it was important to compare the initial rate (v_o) of proteolysis measured for the recombinant full-sized SNAP-25 with those obtained for smaller synthetic substrates. For this purpose, BoNT cleavage of different GST-SNAP-25 concentrations (0.118 to 15μM) in solution were measured via an ELISA, standardised with defined mixtures of BoNT/A-truncated and intact protein detected using anti-SNAP-25_A-pAb (see Materials and Methods). At the highest GST-SNAP-25 concentration (15 μM), BoNT/A gave a v_o of 12.6 ± 1.1 μmoles/min/mg toxin (mean ± S.D.). Analysis of the results using the Lineweaver-Burke plot revealed that saturation of the enzymes active site had not been achieved at the maximum substrate concentration employed (Figure 6). Therefore, both K_m and V_{max} values were unobtainable. The HPLC assay of Schmidt was employed to assess the kinetic parameters of two small synthetic peptide substrates; residues 187-203 (i.e. the substrate optimised by Schmidt and Bostain, 1995; termed acetylated NH 187–203 CA) or a 26-mer peptide residues 181-206 (detailed in 1st Annual Report; also see Figure 7). The kinetic parameters recorded for these three substrates are displayed in Table 1. Both of these short peptides were proteolysed by BoNT/A at initial rates very substantially lower than that observed above for full-length SNAP-25. Previous studies performed by Schmidt and Bostian (1995, 1997) found that this 17-amino acid peptide served as a substrate for BoNT/A; however; extending its length to 66 residues gave much better cleavage (Washborne *et al.*, 1997). Thus, it appears that a 9 residue SNARE motif (145-153), located far upstream of the scissile bond of type A (Rossetto *et al.*, 1994), is essential for efficient recognition by the toxin.

Although 17-mer C-terminal SNAP-25 and the 26-mer (shown herein) are poorly proteolysed by BoNT/A relative to the full-length substrate, the former provides a convenient model for investigating the residues required on either side of the scissile bond. Moreover, site-modified synthetic peptides have the added advantages that they are often much more soluble than large recombinant proteins, allowing use of sufficiently high concentrations to determine the effects of the above modifications on enzymic kinetic parameters. Unfortunately, the apparent low affinities of BoNTs for their optimal substrates (Km between 0.1-2 mM; TeTx, Cornille *et al.*, 1994; TeTx and BoNT/B, Foran *et al.*, 1994) restrict the usefulness of bacterially-expressed recombinant substrates because of the limited concentrations possible.

In view of this discrepancy between the rates of cleavage of the full-length protein and small synthetic peptides, the S4 SNARE motif was evaluated for ability to improve this proteolysis by analogy with TeTx and Sbr domains (Cornille *et al.*, 1997). Unfortunately, a surrogate peptide

(acetylated–NH-ARENEMDENLEQVSG-$CONH_2$) encompassing the S4 SNARE motif of SNAP-25 (see Figure 7) proved ineffective (up to 0.1 mM) in accelerating v_o for BoNT/A proteolyses of either the 26- or 17-mer peptides; at high concentrations, each was inhibitory (IC_{50} ~ 2 mM). Future work will examine if the segment of bacterially-expressed GST-SNAP-25 (residues 1-180), homologous to the BoNT/E-cleaved product, can potentiate the activity of BoNT/A protease towards small substrates because of its S4 SNARE motif being present in the optimal conformation. In addition, the potential inhibitory effects of the S4-SNARE motif peptide on BoNT/A cleavage of the full-sized SNAP-25 was assessed by ELISA; it inhibited BoNT/A proteolysis of full-sized SNAP-25 with an IC_{50} of approximately 0.5 mM (Figure 7).

Production of mutated SNAP-25 genes: their ligation into expression vectors and verification by nucleotide sequencing and restriction digestion.

Several methods were used to introduce single or multiple point-mutations and N- or C-terminal deletions/addition to the SNAP-25 gene. The intended amino acid alterations encoded by the mutant genes are listed in Figure 8. Bold letters indicate the residue(s) changed within the four amino acid sequence that lies between the P2 and the P′2 positions (N- or C-terminal to the proteolysed bond; nomenclature of Schechter and Berger, 1967) in the SNAP-25 molecule. The widely used one- and two-step PCR procedures (reviewed in Higuchi, 1990; also, see Methods) required subsequent ligation of the isolated mutated genes into the pGEX-2T plasmids, whereas the far more rapid *Dpn*-1 nuclease quick-change method (Bergsied *et al.*, 1991; outlined in Methods) allowed modifications of the gene whilst still present within the plasmid; this avoids the aforementioned costly and time-consuming procedures. Initially, one- and two-step methods were employed to create many of the intended mutations (detailed in Figure 8 and listed in Table 2); however, some nucleotide changes failed to be incorporated even after repeated attempts using new batches of primers and varied PCR conditions. Therefore, other mutagenesis methods were sought amongst which the *Dpn*-1 nuclease-quick change appeared to be the most attractive, as it included an additional digestion step enabling recovery of only the plasmid containing the mutated gene. Therefore, subsequent transfections gave rise to transformants with only plasmids containing the mutant (i.e. 100% efficiency), unlike the other methods cited above. However, for addition or removal of nucleotides the one-step PCR method had to be used. An automated fluorescence-based dideoxy-nucleotide termination sequencing method was employed to verify that all of the variant SNAP-25 genes produced had the appropriate nucleotide sequences (data not shown). In the case of the $SNAP\text{-}25_{ADD}$ gene, the additional nucleotides present (from the pGEX

vector encoding 18 additional amino acids at the C-terminus) encode a predicted sequence: $_{(NH2)}$-SEFHRDLTICLARFGDD-$_{(COOH)}$.

Expression and purification of GST-linked mutated SNAP-25 proteins.

The variant GST-SNAP-25 proteins were expressed in E. *coli* and affinity-purified, as outlined earlier for the wild-type; the resultant biospecifically-eluted samples were analysed by SDS-PAGE and immunoblotting. Coomassie staining revealed that all contained protein bands of the appropriate Mr ~ 48 kDa, together with varying amounts of free GST (~ 23 kDa, Figure 9A). As all affinity-purified proteins were eluted in equivalent volumes, it is clear from the varying intensities of the GST-SNAP-25 proteins (Figure 9A) that bacteria expressed these products with somewhat different efficiencies. Additionally, Western blotting with two different affinity-purified anti-SNAP-25 Ig preparations confirmed the identity of the ~ 48 kDa proteins as GST-SNAP-25 fusions (Figure 9B,C). An antibody reactive with the internal C-terminal SNAP-25 sequence (residues 190-197; termed anti-SNAP-$25_{190-197}$), which exhibits equal reactivities with the intact and BoNT/A-truncated molecule, as expected, gave immuno-reactivities (Figures 9, 10) equivalent to the relative abundancies of the proteins visualised by Coomassie staining in most cases (Figure 9A,B). However, only weak reactivity was noted for the triple mutant QAKA and none for the single (NAEA) and triple mutant NAKL (Figure 9B). Furthermore, many of the single and double point mutants reacted with anti-SNAP-25_{FL}-Ig as efficiently as the wild-type molecule (Figure 9C). However, a single alteration (Ala to Leu) in mutant NQRL abolished primary Ig binding, as did the removal of 9 or 8 C-terminal residues in GST-SNAP-25_A and GST-SNAP-25_{C1} (site predicted for BoNT/C1 cleavage), respectively (Figure 9C). Notably, neither of the two triple mutants (QAKA or NAKL) reacted with the latter Ig (Figure 9C).

Western blotting of the affinity-purified, addition and deletion mutants of GST-linked SNAP-25 also revealed bands of the appropriate sizes (predicted from their primary amino acid sequences (listed in Figure 10A). Whilst SNAP-25 proteins modified at their C-termini were efficiently expressed in *E. coli*, only low levels of one of the N-terminal mutants could be obtained, as reflected by the weaker immunoreactive signal and larger amounts of free GST (not shown). Due to the anticipated higher solubility of N-terminally truncated SNAP-25 proteins (89 and 64 amino acids in length) and lower tendencies to aggregate at elevated concentrations than that exhibited by the full-length SNAP-25, these could prove advantageous for future assessments of the kinetic parameters (Km, kcat) of proteolysis by BoNT/A.

Production of mutant SNAP-25 highly resistant to proteolysis by BoNT/A: arginine at position 198 is crucial for efficient cleavage.

The eleven different full-length mutated SNAP-25s generated containing single, double or triple substitutions at P2 through to P′2 positions were examined for susceptibility to BoNT/A protease relative to the wild-type recombinant protein - the most efficient substrate. Earlier studies (Schmidt and Bostain, 1997) examining the importance of SNAP-25 residues P5 through to P′5 for BoNT/A protease found that the P`1 arginine residue at position 198 is critical.

The optimised and standardised ELISA which allows one to quantify the amounts of either full-length SNAP-25 or the BoNT/A-truncated product (residues 1-197; termed SNAP-25_A) was employed for measuring proteolysis of recombinant SNAP-25 by this toxin. Either wild-type (wt) or mutant GST-SNAP-25s were coated at equimolar amounts onto wells (detailed in Materials and Methods). After blocking excess binding sites with BSA, the wells were exposed to increasing concentrations of reduced BoNT/A; the extents of proteolysis were monitored with either anti-SNAP-25-(C-terminus) reactive antibody (Figure 11A,B) or anti-SNAP-25_{A1}-pAb (Figure 11C), depending upon the particular mutant being studied. Use of different antibodies for detection was necessary because alteration of some residues in SNAP-25 protein abolished primary antibody binding. Notably, SNAP-25_{1-202}, A199L, and SNAP-25_{ADD} mutants were not reactive with anti-SNAP-25-(C-terminus)-Ig. Conversely, the numerous mutants described that contained substitutions at position Q197 did not react with anti-SNAP-25_A-pAb after BoNT/A proteolysis due to the loss of this essential epitope. Therefore, anti-SNAP-25-(C-terminus) Ig was used to detect the toxin's cleavage of single mutants altered at the P1 and/or P′1 sites (Figure 11A,B). The relative amounts of wt or mutant SNAP-25s remaining intact following toxin treatment are expressed as A405 nm readings relative to those for toxin-free controls. Incubation of SNAP-25 or its P1/P′1 variants for 6 h at 37°C with very high concentrations of reduced BoNT/A (up to 450 nM) was required to give partial or extensive proteolysis of the single- and double-point mutants. BoNT/A proteolysis of the three remaining SNAP-25 mutants, that lacked reactivity to anti-SNAP-25-(C-terminus) Ig, were assessed by ELISA using anti-SNAP-25_A-pAb (Figure 11C). As before, wells coated with equal amounts of either wt or mutant SNAP-25 were exposed to increasing concentrations of reduced BoNT/A (up to 100 nM) for 90 min at 37°C. After development and subtraction of the A405 nm signal in wells lacking SNAP-25 from those for all samples, the resultant values (means ± S.D.; n ± 4) were plotted. Notably, wt and SNAP-25 mutants all exhibited different susceptibilities to BoNT/A (Figure 11A,B,C). The EC_{50} for the cleavage of wt and each SNAP-25 mutant by BoNT/A was extrapolated from Figure 11 and displayed in Table 2.

It was found that changes at the P′1, but not at the P1 position, yielded proteins highly resistant to BoNT/A compared to the wild-type substrate; replacement of P′1 arginine with either alanine (R198A)

or threonine (R198T) reduced degradation of SNAP-25 by ~ 560- and 16,000-fold, respectively (Table 2). In contrast, alteration of the P1 glutamine to alanine caused little change (Q197A; Table 2). Similar observations highlighting the importance of P`1 substrate residues has also been reported by Shone & Roberts (1994) in the case of BoNT/B cleavage of Sbr peptides. Notably, they also observed that alterations of the nature of the residue at the P1 position in Sbr is less important for BoNT/B proteolytic activity, as found herein for SNAP-25 and type A activity. The rate of hydrolysis by BoNT/A was also strongly affected by substitutions at the P2 position; changing an Ala to a larger hydrophobic Leu residue caused ~50-fold lower susceptibility to BoNT/A-mediated cleavage. Interestingly, Schmidt & Bostian (1997) reported that when an analogous replacement was made in their small substrate, the mutant was capable of binding BoNT/A as efficiently as the wild-type substrate. However, our observation that the A199L mutant is significantly proteolysed by toxin would appear to exclude its usefulness as an inhibitor. On the other hand, the double-point mutations incorporating the innocuous P1 alanine replacement, in addition to alterations at the P′1 site, to either alanine (Q197A/R198A), lysine (Q197A/R198K) or glutamic acid (Q197A/R198E) produced even lower susceptibilities to proteolysis by type A toxin (~4,400-, 38,000- and 38,000-fold, respectively). Unexpectedly, the double-point SNAP-25 mutant Q197A/R198A which shared the Gln to Ala substitution at the P1 position (a single mutation that has little effect on BoNT/A activity alone) but that also includes an Ala substitution at the P′1 residue, was much more resistant to the toxin (i.e. approximately 4400-fold lower susceptibility to BoNT/A protease) compared to the single P′1 mutant containing the R198A alteration (i.e. ~560-fold slower). The underlying reason for this anomaly is uncertain, but may reflect a conformational change in the substrate at the cleavage site. Therefore, point mutations may significantly alter the conformation of the C-terminus of SNAP-25, as far as this protease is concerned. Other double-point mutations at the P1 and P′1 positions [i.e. the Q197/R198 sequence to either KH [Q197K/R198H; naturally occurring in *Torpedo* SNAP-25 (Washbourne *et al.*, 1997) or WW (Q197W/R198W)] caused less resistance to BoNT/A (~ 440- and 96-fold, respectively; Table 2). The weak susceptibility of *Torpedo* electric organ SNAP-25 to BoNT/A, an important issue because this is a strictly cholinergic tissue and useful experimental model which shares many similarities to the mammalian NMJ. The unexpectedly modest reduction in susceptibility observed upon changing the P1 and P′1 positions in SNAP-25 from Q-R to W-W suggests that the BoNT/A enzyme S`1 sub-site favours aromatic residues to some extent. In order to confirm this possibility, extra experiments are required involving single point P`1 mutations of the wt Arg to either Trp, Phe or Tyr. During the course of this study, others (Vaidyanathan *et al.*, 1999) reported a similar high resistance of SNAP-25 to scission by BoNT/A upon incorporation of P′1 replacements.

In addition to the above changes, other novel changes have been inserted into the SNAP-25 protein. Whilst removal of four residues resulted in only minor alteration in susceptibility to the protease, addition of 18 residues had a moderate inhibitory effect (Table 2). In the latter case, addition of 18 amino acids to the C-terminus of SNAP-25 (sequence added, SEFHRDSLTICLARFGDD; derived from the pGEX vector) reduced susceptibility to BoNT/A proteolysis by ~7-fold. Based on this information, the possibility exists of adding a seven amino acid C-terminal biotin-mimic tag (available commercially) to SNAP-25 enabling direct measurement of BoNT/A cleavage with enzyme-linked streptavidin. Development of such an assay would provide a more rapid assay compared to the indirect two-step antibody-based approach currently being employed, and eliminate the need to generate highly specific Ig preparations.

The lack of immunoreactivities of both triple mutants towards either anti-SNAP-25-(C-terminus) Ig or anti-SNAP-25_A-Ig precluded the ELISA-based assessment of their susceptibilities to proteolytic attack by BoNT/A. Therefore, their susceptibility to cleavage by BoNT/A was assessed, following a 2 h incubation at 37°C with different concentrations of reduced toxin, by SDS-PAGE and visualisation of protein by silver staining. Whilst the wt substrate was completely proteolysed by the lowest concentration of BoNT/A tested (Figure 12A; 0.1 nM), the small shift in Mr seen with the latter was not detectable for either N196Q/Q197A/R198/K (Figure 12B) or Q197A/R198K/A199L mutants (Figure 12C) when up to 100 nM toxin was used. Although the latter method is prone to possible errors, it appears that these triple mutants are also highly resistant to attack by the toxin.

Different time courses for recovery of evoked secretion from poisoning with BoNT/A or B: a process coincident with reappearance of intact SNARE.

To investigate the important question of how BoNTs exert their inhibitory actions for prolonged periods, neuroendocrine adreno-chromaffin cells were intoxicated for 24 h with BoNT/A (6.6 nM) or B (66 nM) using a low-ionic strength medium (LISM), to overcome the absence of high affinity acceptors (Lawrence *et al.*, 1996). Evoked secretion and SNARE contents were assessed at regular intervals on days 5, 19, 40 and 56, post intoxication (Figure 13). Ba^{2+} was employed instead of other common stimuli (e.g. 55 mM K^+ or nicotine that induce Ca^{2+} entry) because it evokes catecholamine release up to 50% of the total content compared to only ~20 % by the latter stimuli, while still exhibiting the same SNAP-25 and Sbr/Cbr requirements as that induced by Ca^{2+} (Lawrence *et al.*, 1996). As expected, BoNT/A or /B treatments at day 5 post-intoxication both yielded extensive inhibition of Ba^{2+}-evoked catecholamine release of 84 ± 0.7% and 90 ± 0.4% (means ± S.E.; n=4), respectively, relative to that for toxin-free controls. Intact SNAP-25 was monitored using anti-SNAP-25(C-terminus)-IgG, whilst intact Sbr and Cbr (a close BoNT/B-sensitive homologue (McMahon *et al.*, 1993) was assessed using

anti-Sbr/Cbr-IgG. Both of these antibody preparations were un-reactive towards the BoNT-cleaved products. Further, the presence of BoNT/A-truncated SNAP-25 was assessed using anti-SNAP-25_{A1}-pAb. This antibody was unreactive with intact SNAP-25 in neurotoxin-free cells, but reacted strongly with the truncated product in BoNT/A poisoned cells (Figure 13; and as found previously by Lawrence *et al.* (1997). The BoNT-mediated inhibition of secretion noted at day 5 was accompanied by total or near-complete cleavage of intact SNAP-25 or Sbr/Cbr (Figure 13). In addition, the BoNT/A-truncated SNAP-25 was present at this time (Figure 13A). At day 40 post-intoxication, a trace of intact SNAP-25 was observed in BoNT/A-poisoned cells; however, the amount noted was much lower than normal and insufficient to significantly influence the level of evoked secretion recorded compared to day 5 (Figure 13A). However, at day 56 post-intoxication, slightly larger amounts of intact SNAP-25 were noted in BoNT/A poisoned cells, apparently enough to cause a statistically significant, but partial, recovery of exocytosis, equivalent to a doubling of the amount of Ba^{2+} evoked catecholamine release from 16 to 34%. Throughout this time course, $SNAP\text{-}25_A$ persisted in intoxicated cells (Figure 13A). Difficulties inherent to primary cell cultures precluded studies longer than eight weeks post-intoxication, periods that would appear to be necessary to attain complete recovery from BoNT/A.

Conversely, 56 days was sufficient to allow near-complete recovery from BoNT/B-poisoning. The inhibition of evoked secretion in BoNT/B-poisoned cells initially noted on day 5 post-intoxication of 90 ± 0.4%, gradually subsided until only 10 ± 3.4% inhibition (means ± S.E.; n=4) remained at day 56. Recovery of evoked secretion in BoNT/B-treated cells was coincident with reappearance of intact Sbr/Cbr to a level comparable to that observed in toxin-free cells (Figure 13B).

These different recovery periods for exocytosis in BoNT/A- and B- poisoned chromaffin cells are analogous to those found after focal intramuscular injection of either toxin in humans (Sloop *et al.*, 1997). Here, it is shown for the first time that the near-complete recovery after BoNT/B poisoning is due to appearance of intact Sbr/Cbr. Notably, the time course for the latter correlates with that found for chromaffin cells poisoned with TeTx (Bartels *et al.*, 1994), which suggests that either the turnover of BoNT/B and TeTx are very similar and/or the rate of replenishment of intact Sbr/Cbr limits the resumption of catecholamine release. The much slower rates for recovery, reappearance of newly-synthesised intact SNAP-25, and disappearance of the truncated product in BoNT/A-treated cells show that either this serotype remains active for longer than BoNT/B and/or the turnover of SNAP-25 is significantly slower than that of Sbr/Cbr.

Expression of wild-type and mutant SNAP-25 in CHO cells.

Correct expression of recombinant SNAP-25 from the pcDNA1.1/Amp-SNAP-25 constructs was assessed in CHO cells, since they lack endogenous SNAP-25, using immunoblotting. Anti-SNAP-25(recombinant)-IgG, which recognises both the full-length and BoNT-truncated SNAP-25s, detected

expression of SNAP-25 in cells transfected with the wild-type, mutant R198T or SNAP-25_A mammalian expression constructs (Figure 14). Moreover, recombinant SNAP-25 proteins exhibited the appropriate Mr compared to the native protein present chromaffin cells (Figure 14). Notably, both wild-type and R198T SNAP-25 proteins from toxin-free cells reacted with anti-SNAP-25(C-terminus)-IgG unlike SNAP-25_A; instead, the latter was solely recognised by anti-SNAP-25_{A1}-pAb (Figure 14). In addition, mammalian-expressed SNAP-25s exhibited equivalent sensitivities to BoNT/A protease as the bacterial-generated proteins. Immunoblotting using anti-SNAP-25(C-terminus)-IgG revealed proteolysis of the majority of the wild-type by BoNT/A (leading to the appearance of the SNAP-25_A immunoreactivity), whilst the protease-resistant SNAP-25 R198T mutants reactivity was not altered (Figure 14). Notably, both wt and R198T mutant SNAP-25s were expressed at equivalent levels in transfected cells, using the same transfection protocol. As expected, immunoreactivity for SNAP-25_A in CHO cells was unaffected by BoNT/A treatment (Figure 14). Having demonstrated that the R198T mutant was resistant to BoNT/A cleavage in mammalian cells, plasmids encoding wild-type or this mutant SNAP-25 were introduced into chromaffin cells.

Effects of expression of SNAP-25 on evoked exocytosis from control or BoNT/A-pre-treated chromaffin cells.

It was considered a possibility that introduction of SNAP-25 into BoNT/A-poisoned cells should restore regulated exocytosis by replacement of the endogenous SNAP-25_A. To investigate the effects of introduced SNAP-25 on evoked secretory function in chromaffin cells, the protein was transiently co-expressed with hGH. The latter, which is normally absent from cells, co-localises with catecholamines in LDCVs (Holz *et al.*, 1995). Thus, evoked hGH secretion serves as an excellent reporter for LDCV exocytosis (Figure 15). In control or BoNT/A-treated cells that had been transfected with vectors encoding hGH, and the non-toxic protein CAT, approximately equivalent amounts of Ba^{2+}-evoked hGH and catecholamine secretion were observed (Figure 13A, 15A). Transfection with a plasmid encoding SNAP-25 did not alter the amount of hGH secreted from toxin-free cells; significantly, over-expressed SNAP-25 was unable to overcome the extent of inhibition due to BoNT/A poisoning (Figure 15A). These results suggested that the persistence of BoNT/A maintained the blockade of release.

Effects of expression of SNAP-25_A on evoked exocytosis from control or BoNT/A-pre-treated chromaffin cells.

Having established that wild-type or mutant SNAP-25 are properly expressed, it was deemed important to determine whether the introduced SNAP-25 was participating in exocytosis. Importantly, expression of SNAP-25_A (i.e. analogous to the BoNT/A-cleaved product) in toxin-free cells inhibited evoked hGH

secretion by > 90% of control in a manner similar to the BoNT/A pre-treatment. Expression of SNAP-25_A in BoNT/A pre-poisoned cells consistently abolished the residual evoked secretion (~ 10 % of total) that commonly remained following toxin treatment (Figure 15A). Thus, not only does introduced SNAP-25_A replace the endogenous protein, the displacement of the intact SNAP-25 with the BoNT/A-truncated product establishes that BoNT/A poisoning is due solely to cleavage of SNAP-25.

Rescue of evoked exocytosis in BoNT/A-pretreated chromaffin cells by expression of SNAP-25 mutants resistant to the toxin : validation of the recovered secretion by its inhibition with BoNT/E.

After demonstrating the validity of the system, transfection of plasmids directing the expression of BoNT/A protease-resistant SNAP-25s were performed. Introduction of SNAP-25 mutants into toxin-free chromaffin cells caused only a small reduction in the ability to secrete hGH, as compared to controls (Figure 15A). As hoped, expression of each protease-resistant SNAP-25 in BoNT/A-treated cells (in which catecholamine release is inhibited; data not shown) almost completely rescued secretory function close to the levels recorded for toxin-free controls (Figure 15A). This experiment was repeated several times, with the pattern being the same in each case. Overall, we have established that expression of BoNT/A protease-resistant SNAP-25s can efficiently rescue secretory function in cells poisoned 5 days earlier.

An additional experiment was performed to conclusively demonstrate that the rescue of evoked hGH secretion in BoNT/A pre-intoxicated cells was a direct result of the participation of expressed BoNT/A protease-resistant SNAP-25. Digitonin-permeabilisation was used to introduce BoNT/E into cells (because the LISM protocol is ineffective for BoNT/E), so that this toxin could further proteolyse the artificially-expressed BoNT/A protease-resistant SNAP-25 R198T mutant, thus, negating its protective effect. As expected in cells lacking protease-resistant SNAP-25, prior BoNT/A poisoning caused an 82 ± 4.3% inhibition of hGH release when compared to toxin-free cells. On the other hand, evoked hGH secretion in BoNT/A pre-intoxicated cells that expressed the SNAP-25 protease-resistant R198T mutant was largely protected. Importantly, when BoNT/A-pre-treated cells expressing R198T mutant were permeabilised and exposed to reduced BoNT/E (using conditions known to proteolyse nearly all SNAP-25 (Lawrence *et al.*, 1997), the partial rescue of evoked secretion in BoNT/A-poisoned cells (noted above) was largely abolished, and an 88 ± 7.8% inhibition of evoked hGH secretion was recorded (Figure 15B). This experiment was repeated twice, with the pattern being the same in each case.

The ability of BoNT/A-resistant SNAP-25 to rescue release provides some important insights about SNAP-25 and its BoNT/A cleavage site. First, over-expression of either wild-type SNAP-25 or

BoNT/A-resistant mutants do not seem to cause toxicity to the cell, since the characteristics of release from the toxin-free cells appear to be unaffected. In addition, recombinant SNAP-25 has been previously shown to be targeted to the appropriate plasma membrane locations in PC-12, as well as insulinoma cells (Bark *et al.*, 1995; Gonelle-Gispert *et al.*, 1999). As SNAP-25 must complex with the limited amounts of syntaxin1 and Sbr present in order to exert its co-operative exocytotic function, it is reasonable to assume that most of the excess protease-resistant SNAP-25s competes out the native SNAP-25 for SNARE complex formation. The fact that BoNT/E inhibits this rescued exocytosis provides further proof for the participation of BoNT/A-resistant SNAP-25 in SNARE-mediated exocytosis. Realising these important considerations, it is evident that residues Q197 and R198 in SNAP-25, which were altered in the protease-resistant mutants, are not essential for evoked exocytosis. These studies and those of others have shown that there is a high degree of amino acid degeneracy in SNAP-25 (O'Sullivan *et al.*, 1999). When the BoNT/E cleavage site (residues 180-181) in SNAP-25 was mutated, it was found that over-expression of BoNT/E-resistant SNAP-25 prevented the anticipated inhibition of evoked secretion upon exposure to BoNT/E (Gonelle-Gispert *et al.*, 1999), which shows that these residues are also not critical for exocytosis. Further, a recent site-directed mutagenesis study of SNAP-25 has revealed that mutations of hydrophobic residues, found within the core of the four-helix bundle, significantly limited rescue after BoNT/E poisoning (Chen *et al.*, 1999). The full extent of this degeneracy can be seen when human SNAP-23 (a BoNT/E-resistant non-neuronal homologue which is only ~60 % identical to SNAP-25) is expressed in permeabilised insulinoma cells; this supported the same extent of exocytosis upon treatment with BoNT/E as that found for control cells (Saduol *et al.*, 1997). However, our study is the first to report the amino acid degeneracy of SNAP-25 by quantifying the abilities of BoNT/A-resistant mutants to rescue exocytosis in intact BoNT pre-poisoned cells.

Three weeks after exposure to BoNT/A, only protease-resistant SNAP-25 rescues regulated exocytosis: demonstration of the persistence of active toxin protease.

The inability to achieve rescue of exocytosis upon transfection of wild-type SNAP-25 into BoNT/A-poisoned cells suggested that the activity of the toxin persisted for at least a week in these cells (Figure 15A). To determine how long BoNT/A remained active, this experiment was repeated up to 3 weeks after exposure to BoNT/A (Figure 16). As expected, toxin-free cells transfected with hGH and CAT encoding control plasmids (i.e. recombinant SNAP-25-free control), released hGH in a divalent cation-dependent manner (Figure 16). Cells exposed to BoNT/A 3 weeks previously, when transfected with the same vectors gave a much-reduced amount of evoked secretion (Figure 16). Therefore, toxin pre-treatment still afforded a large extent of blockade even after three weeks. As before, expression of

wild-type SNAP-25 did not significantly alter the level of evoked hGH secretion occurring in toxin-free cells and, significantly, failed to overcome the blockade in toxin-treated cells (Figure 16). It seems likely that the newly-expressed wild-type SNAP-25 was cleaved by active BoNT/A protease persisting in the cells. Importantly, the extent of evoked hGH secretion recorded from cells intoxicated three weeks earlier expressing protease-resistant SNAP-25 R198T was returned to the normal level (see above; Figure 16). This experiment was repeated four times, with the pattern being the same in each case. Hence, we show that BoNT/A is proteolytically active for at least three weeks after poisoning. This prolonged activity suggests that, at least in chromaffin cells, the continued activity of BoNT/A-LC plays a key role in the continued inhibition of release. Moreover, it is likely that toxic amounts of active BoNT/A protease remain in cells even 56 days post-exposure and continues to do so until the eventual complete recovery of evoked secretory function (not determined). Whilst this agrees with previous data published on chromaffin cells (Bartels *et al.*, 1994), it seems to contradict some more recent findings. Human muscles at the NMJ double-poisoned by injection with a mixture of BoNT/A and /E recovered as if they had been solely intoxicated with BoNT/E, which has a shorter time-course for recovery than A (Eleopra *et al.*, 1998). These results suggest it is not the continued BoNT/A activity but the persistence of SNAP-25_A that limits recovery. However, it is unclear how the removal of 17 extra amino acids from the C-terminal end of SNAP-25_A leads to a more rapid recovery of neurotransmission. It is likely that this apparent contradiction between results in chromaffin cells (our data and Bartels *et al.*, 1994) and those found at the NMJ is due to a different ratio of SNAP-25 synthesis to BoNT/A-LC activity but this remains to be determined.

Identification of the C-terminal amino acids of SNAP-25 necessary for exocytosis.

When BoNT/A-truncated SNAP-25, which had been shown to be expressed in CHO cells (Figure 14), was introduced into chromaffin cells, it diminished evoked hGH release (Figure 15A). Moreover, its expression in BoNT/A-prepoisoned cells consistently diminished the residual evoked secretion of hGH that commonly remained following toxin treatment (Figure 15A). Thus, it appears that the endogenous intact SNAP-25 can be displaced from the SNARE complex by SNAP-25_A; hence, this product contributes to the inhibition of exocytosis by BoNT/A. Next, it was pertinent to determine how many of the C-terminal residues of SNAP-25 need to be added back to restore its role in exocytosis; this was addressed using a series of truncated, toxin-resistant mutants illustrated in Figure 17B. Recent structural data on the SNAP-25 C-terminal domain (Poirier *et al.*, 1998) suggests that when it participates in the core complex residues 141-206 form a helical structure in which one complete turn involves seven residues, labelled 'a' to 'g'(Figure 17B). Identification of the maximum number of residues that can be removed from the C-terminal end of SNAP-25, without affecting its ability to

rescue exocytosis, would indicate where this functional domain terminates. In this regard, it is noteworthy that the expression of SNAP-25_{1-198} [the product of cleavage with BoNT/C1 and known to be resistant to BoNT/A (data not shown)], SNAP-25_{1-199}-R198T or SNAP-25_{1-200}-R198T inhibited secretion in toxin-free or -treated cells relative to that seen with full-length SNAP-25-R198T (Figure 17A). In contrast, a normal level of secretion was observed with SNAP-25_{1-202}-R198T or SNAP-25_{1-203}-R198T in toxin-free cells; moreover, all of these BoNT/A-resistant mutants rescued release in BoNT/A pre-poisoned cells (Figure 17A). The noted ability of SNAP-25_{1-201}-R198T to support exocytosis to a significant but lower level than SNAP-25_{1-206}-R198T suggested that the R198T mutation might compromise slightly the ability of SNAP-25 to function in exocytosis which only becomes apparent in this shortened construct. Indeed, this seems to be the case because the wild-type R^{198} version of SNAP-25_{1-201} gave the control level of release, when introduced into non-poisoned chromaffin cells. To conclusively prove that residues 197 to 201 are sufficient for normal exocytosis, the adjoining M^{202} was mutated to an alanine in full-length SNAP-25; significantly, SNAP-25_{1-206}-M202A preserved the full complement of hGH secretion (Figure 17A). Further, expression of the BoNT/A-resistant SNAP-25_{1-206}-R198T/M202A not only supported exocytosis in non-intoxicated cells but it rescued secretion in poisoned chromaffin cells (Figure 17A). Thus, it seems that wild-type SNAP-25 encompassing residues 197-201 can mediate exocytosis without M^{202} or the last 4 residues. As a recent study (Criado et al., 1999) indicated that mutating L^{203} to alanine in wild-type SNAP-25 suppressed exocytosis in chromaffin cells, this point mutant was evaluated. Introduction of SNAP-25_{1-206}-L203A into chromaffin cells caused no inhibition of hGH secretion (Figure 17A); further, expression of its BoNT/A-resistant variant, SNAP-25_{1-206}- R198T/L203A, mediated exocytosis in non-intoxicated cells and afforded near-complete rescue of secretion after poisoning (Figure 17A). This discrepancy may relate to the use of SNAP-25 mutants tagged with Green Fluorescent Protein (GFP) in the other study .

BoNT/A-induced blockade of neurotransmitter release in cerebellar neurons persists for many weeks: co-incident with continued proteolysis of SNAP-25.

To gain further molecular insights into the prolonged neuroparalysis observed with BoNT/A at the NMJ, biochemical investigations were undertaken to measure the life-time of BoNT/A in neurons, which would be more representative of motor nerves than the chromaffin cells used earlier. Rat cerebellar granule cells provided a convenient model of homogeneous neurones for such studies on toxin-induced transmitter release and SNAP-25 cleavage. Treatment of these cultured neurons with 5pM BoNT/A for 24 h inhibited 80% of the component of Ca^{2+}-dependent K^{+}-evoked transmitter release inhibitable by the toxin (see legend Figure 18) and, concomitantly, cleaved the majority of

SNAP-25 present (Figure 18B). Importantly, no recovery of release or replenishment of intact SNAP-25 was detected after 31 days following toxin removal (Figure 18A), establishing that the protease must persist for an inordinate time and, possibly, there is slow removal of SNAP-25_A. Pulse-chase experiments with [^{35}S]-methionine, and immunoprecipitation of newly-synthesised labelled SNAP-25, in neurons pre-poisoned with BoNT/A unambiguously revealed (Foran *et al.*, 2001) that the protease remains able to produce SNAP-25_A for 15 days (Figure 18C), in fact, more than 3 weeks (not shown)]. This amazing longevity of type A toxin, demonstrated directly in this way for the first time, accords with the lack of recovery of neuro-exocytosis and the aforementioned results of rescue experiments on neuroendocrine cells, as well as the prolonged cleavage seen in spinal cord cultures (Keller *et al.*, 1999).

BoNT/E causes a short-lived appearance of SNAP-25_E and inhibition of transmitter release from cerebellar neurons : co-poisoning with BoNT/E and /A fails to shorten the prolonged blockade of exocytosis caused by the latter and the predominance of SNAP-25_A.

To help gain insight into the molecular basis of this persistent neuroparalysis by BoNT/A, cerebellar neurons were treated with type E toxin which produces a more truncated fragment (residues 1-180) of SNAP-25 (Figure 19). This virtually abolished evoked transmitter release and gave a corresponding extent of cleavage of SNAP-25; the identity of its truncated product was confirmed with IgG reactive exclusively with SNAP-25_E (data not shown). Notably, the BoNT/E-induced blockade of glutamate exocytosis was found to be short-lived, with an almost complete recovery seen within only 5-7 days accompanied by a disappearance of SNAP-25_E (Figure 19). The contrasting sustained action of BoNT/A and the transient effect of /E (Figure 19) mimic the patterns observed at the NMJ, thereby, validating the suitability of these cultured neurons as a convenient model for future assessment of our novel rescue strategies for toxin poisoning. Co-poisoning of the neurons with both BoNT/A and /E yielded essentially only SNAP-25_E (i.e. SNAP-25_A formed was efficiently proteolysed by type E). Most importantly, the E-truncated SNAP-25 was quickly degraded and SNAP-25_A reappeared so neuro-exocytosis failed to recover past the type A inhibited level at 15 days later (Figure 19). These pertinent observations indicate that the longevity of the BoNT/A protease, together with an apparent slow replacement of SNAP-25_A, are contributory factors to the prolonged intoxication of these particular neurons. Hence, it is clear that useful therapy for botulism necessitates strategies that can provide full-length functional SNAP-25 over long periods, as well as a means of inhibiting the protease.

Highly efficient transduction of cultured neurons using recombinant replication-incompetent adenovirus.

Effective application of our proven replacement of BoNT/A-resistant SNAP-25 as a therapy for botulinisation requires very effective transfection methods for neurons. As adenoviral vectors are known to efficiently transduce neurons in culture or *in situ* for the necessary prolonged periods (Davidson *et al.*, 1993; Robert *et al.*, 1997; Chen et al., 1997) and lack the toxicity associated with other neurotrophic viruses [e.g. Herpes simplex (Kaplitt and Makimura, 1997; Slack and Miller, 1996)], we tested a replication deficient-adenovirus Ad 5 containing a β-galactosidase (β-gal) reporter with a nuclear targeting N-terminal tag (gift from Dr. Andy Knight, Imperial College; Schneider *et al.*, 2000). When this was applied to rat cerebellar granule neurons in culture, highly efficient transfection resulted. Maximal transfection (> 90%) of the neurons occurred within 2 days of viral application, as revealed by staining for β-gal activity and visualisation in the light microscope (Figure 20). Importantly, such exposure to the virus did not alter the extent of transmitter release or cause neuron death.

Subcloning of wild-type Q198T mutant, SNAP-25$_A$, SNAP-25$_E$ and green fluorescent protein into a adenoviral DNA vector and generation of replication-deficient virus.

Encouraged by this success, a recently-launched variant Adeno-X Tet-Off system (Clontech) was adopted because of its safety features (outlined below) and affording the added advantage of controlling the extent and time of SNARE expression – a feature essential for both optimizing the new therapies and for the proposed mechanistic studies. The system (shown diagrammatically in Figure 21) utilizes a shuttle vector that allows rapid insertion (relative to the earlier very time-consuming homologous recombination) of the gene of interest; it has the advantages of being small (3.9 kB), easy to manipulate and high copy number. This step is followed by excision of its sequence together with the CMV Tet response element (TRE) promoter and unidirectional sub-cloning into the Adeno-X viral DNA. After its propagation in *E. coli*, the DNA is linearised and used to transfect HEK 293 cells; for safety, the E1 and E3 elements essential for viral replication have been deleted and, thus, the virus can only replicate in this cell line engineered to provide that protein. In addition to replication being restricted in this way, another level of safety is in-built by the necessity to add a second virus, termed the Adeno-X Tet-Off regulatory component containing the gene encoding the Tet transactivator protein, which activates the CMV TRE promoter in the Adeno-X TRE virus and allows expression of the gene product.

Thus, the cDNAs encoding mouse wild-type SNAP-25b, Q198T mutant, SNAP-25_A, SNAP-25_E and green fluorescent protein (GFP: a non-toxic visual marker) contained in the pcDNA1.1 vector were digested with *Bam* H1 and *Not* 1 and gel purified (Figure 21A). This treatment allowed unidirectional cloning of all of the genes into the similarly pre-digested pTRE-shuttle vector. Ligation was confirmed by restriction digest analysis of plasmid DNA isolated from *E. coli* transformants, carrying resistance to kanamycin (Figure 21B). The SNAP-25 or GFP genes were then excised from the shuttle vector in the form of a DNA cassette containing the CMV-TRE promoter, by using two unique enzymes (PI-*Sce* 1 and I-*Cue* 1; Figure 21C), and ligated into the much larger (~ 31 kB) pre-cut Adeno-X viral DNA (Figure 21D). The resulting ligated cassette containing Adenoviral DNA was then digested with *Swa* 1 to eliminate non-recombinants and used to transform *E. coli.* Kanamycin-resisitant recombinants were selected and screened by PCR, using primers selective only for viral DNA containing the cassette. Transformants were amplified and plasmid DNA purified. Initially, much of the large adenoviral DNA (~ 35 kB) purified was found to have undergone recombination and truncation in the *E. coli* strain provided (not shown). This problem was overcome by using an alternate strain containing a mutation in the recA gene (termed STBL2TM; GIBCO BRL, UK) that enables stable maintenance of large DNA vectors. DNA sequencing and restriction digest analysis confirmed the successful generation of viral DNA containing the genes of interest. Prior to transfecting HEK 293 cells (Figure 21E), the DNA had to be linearised using Pac 1.

Following transfection of linearised DNA into HEK-293 cells and several cycles of viral propagation (see Methods), useful amounts of ~4 x 10^9 and ~3 x 10^9 plaque forming units (p.f.u.) of both GFP-expressing TRE and Adeno-X Tet-Off regulatory viruses, respectively were generated from ~ 3.5 x 10^7 infected cells. Having generated the necessary Adeno X viral DNA constructs containing the other SNAP-25 genes in sufficient quantities, these are currently being transfected into HEK 293 cells, in order to produce replication-incompetent adenoviral particles.

Successful infection of peripheral neurons using the Adeno-X Tet-Off system in conjunction with our generated GFP-TRE adenovirus: detection by appearance of GFP fluorescence.

As a prerequisite to using the appropriate SNAP-25-expressing viruses for the rescue of neurons from BoNT/A poisoning, the GFP-expressing TRE adenovirus was applied to dorsal root ganglionic neurons in culture, together with the Adeno-X Tet-Off regulatory virus in order to demonstrate its usefulness for our intended purpose and to determine the optimal conditions required for maximal protein expression.

In pilot experiments, co-infection of rat dorsal root ganglion neurons with both viruses (Figure 22) and CHO cells (not shown) resulted in expression of GFP when tetracycline was absent, as visualised by microscopy comparing phase contrast and fluorescent images. Such progress has

provided proof of principle for the applicability of this sophisticated and efficient technology for regulated protein expression in neurons. Thus, similar production of SNAP-25 variants also resistant to BoNT (as outlined above) is being pursued as a pre-requisite to evaluating their abilities to rescue transmitter release from poisoned nerves.

KEY RESEARCH ACCOMPLISHMENTS

1. For the quantitation of BoNT/A-induced proteolysis of full length SNAP-25 (determined herein to be the best substrate; see 5 below) a rapid, sensitive and standardized ELISA was established, using several unique anti-peptide antibodies generated for this study.
2. The susceptibilities of 13 different single, double and triple point mutants of SNAP-25 (generated as outlined in 1st Annual Report) to proteolysis by BoNT/A have been assessed by ELISA.
3. The P'1 arginine of SNAP-25 is the most important for defining efficient cleavage by BoNT/A.
4. Additional double- and triple-point mutations of SNAP-25 raised the resistance to BoNT/A up to ~40,000-fold relative to the wt substrate.
5. BoNT/A proteolyses full-sized SNAP-25 more efficiently (~ 100 fold faster at 15 μM) than smaller peptide substrates (17 or 26 amino acids in length) which lack the S4 motif (residues 145-155).
6. A surrogate peptide encompassing the S4-motif of SNAP-25 proved ineffective in accelerating BoNT/A proteolysis of the above-noted synthetic peptides or full-length SNAP-25, unlike tetanus toxin's proteolysis of Sbr.
7. BoNT/A exerts a prolonged intoxication of adreno-chromaffin cells that is accompanied by complete cleavage of SNAP-25 and continues for at least 2 months (the maximum period studied), presumably due to the persistence of cleaved SNAP-25 (residues 1-197).
8. Exocytosis in BoNT/B-treated cells, initially inhibited completely due to cleavage of Sbr and Cbr targets, resumed in full after 2 months; this was accompanied by the appearance of normal amounts of the intact target proteins.
9. The reported time courses for recovery from neuromuscular paralysis following injection of BoNT/A or /B into patients parallels that found herein, validating the chromaffin cell model.
10. BoNT/A poisoning of regulated catecholamine release from neuro-endocrine chromaffin cells (a process that shares many of the properties of neurotransmitter release from neurons) can be quickly and completely rescued through the introduction of any of five BoNT/A-resistant SNAP-25s.
11. As expression of BoNT/A resistant, but not wt, SNAP-25 rescues catecholamine release in chromaffin cells that have been poisoned for at least 3 weeks, the LC protease appears to remain active for prolonged periods.

12. The seven C-terminally truncated forms of SNAP-25 generated have helped define the minimal essential C-terminus required to support exocytosis (i.e. 1-201); the latter fragment is also more resistant to BoNT/A protease.
13. The N-terminally truncated SNAP-25s generated herein will, undoubtedly, aid future determination of the size required for optimal rescue of exocytosis.
14. As a prelude to adapting our proven strategy for rescue from BoNT/A-poisoning of chromaffin cells to the neuromuscular synapse, we have further characterised the nature of the toxins' blockade of transmitter release using closely related and amenable cerebellar neurons. Following intoxication, extensive inhibition was observed together with near complete cleavage of SNAP-25. Importantly, no significant recovery from this blockade of transmitter glutamate exocytosis or reappearance of intact SNAP-25 could be detected 31 days after initial exposure to 5 pM toxin.
15. [^{35}S]-Methionine-labeling and immunoprecipitation of SNAP-25 from neurons poisoned 3 weeks previously, revealed that the LC protease also persists in neurons for prolonged periods; thus, a useful therapy for botulism must replenish the functional SNAP-25 over long periods.
16. Highly efficient transfections (> 90%) of neurons in culture have been accomplished using replication-deficient adenoviral vectors, thus, identifying an essential technology necessary for future application of our rescue strategy to botulinised neurons.
17. The cDNAs encoding mouse wild-type SNAP-25b, Q198T mutant, SNAP-25$_A$, SNAP-25$_E$ and green fluorescent protein (GFP: a non-toxic visual marker) have been incorporated into the Adeno-X TRE viral DNA of the recently-launched Adeno-X Tet-Off system.
18. Sufficient amounts of GFP-expressing and Tet-Off regulatory adenovirus have been generated enabling efficient transduction of peripheral neurons in culture, thereby, validating this transfections method.
19. Adequate amounts of viral DNA for the SNAP-25 expressing constructs have now been produced and are being transfected into the complementary virus-packaging cell line, to produce adenoviral particles for the rescue of neurons from BoNT/A poisoning.

Additional Advances : Cholinergic targeting and intra-neuronal delivery of therapeutic molecules and expression vectors has been made possible by our successful recent development of single chain technology to express atoxic BoNT/E as a single protein in a correctly-folded fully-soluble form that can be converted into the functional transporter by nicking *in vitro*. This major advance has overcome the problems of preparing soluble HC and enzymically-inactive LC of BoNT, and the low yield of reconstituted disulphide-linked transporter.

REPORTABLE OUTCOMES

Publications

1. Li, Y., Foran, P.G., Lawrence, G.W., Mohammed, N., Chion, C.C.K., Aoki, R and Dolly, J.O. (2001) Novel recombinant single-chain tetanus toxin activatable by nicking : a protease-inactive mutant potentially antagonises the native toxin (Submitted).

2. Foran, P.G., Mohammed, N., Lisk, G., Lawrence, G.W. and Dolly, J.O. (2001) Molecular basis for dissimilar durations of blockade of neuro-exocytosis by botulinum toxin A, B, C, E and F (Being submitted).

3. Ashton, A. C. and Dolly, J. O. (2000) A late phase of exocytosis from synaptosomes induced by elevated $[Ca^{2+}]_i$ is not blocked by *Clostridial* neurotoxins. *J. Neurochem.* **74,** 1979-1988.

4. O'Sullivan, G.A., Mohammed, N., Foran, P.G., Lawrence, G.W. and Dolly, J.O. (1999) Rescue of exocytosis in botulinum toxin A-poisoned chromaffin cells by expression of cleavage-resistant SNAP-25: identification of the minimal essential C-terminal residues. *J. Biol. Chem. 274,* 36897-36904.

5. Foran, P. G., Fletcher, L. M., Oatey, P. B., Mohammed, N., Dolly, J. O. and Tavare, J. M. (1999) Protein kinase B stimulates the translocation of GLUT4 but not GLUT1 or transferrin receptor in 3T3-L1 adipocytes by a pathway involving SNAP-23, synaptobrevin-2 and/or cellubrevin. *J. Biol. Chem.* **274,** 28087-28095.

6. Li, Y, Aoki, R. and Dolly, J.O. (1999) Expression and characterisation of the heavy chain of tetanus toxin: reconstitution of the fully-recombinant dichain protein in active form. *J. Biochem.* **125,** 1200-1208

7. de Paiva, A., Meunier, F.A., Molgo, J., Aoki, R. and Dolly, J. O. (1999) Functional repair of motor endplates after botulinum toxin-A poisoning: Bi-phasic switch of synaptic activity between nerve sprouts and their parent terminals. *Proc. Natl. Acad. Sci.* (USA) **96,** 3200-3205.

8. Dolly, J.O. Therapeutic and research exploitation of botulinum neurotoxins. (1997) *Eur. J. Neurol.* **4,** S5-S10.

9. Lawrence, G.W., Foran, P., DasGupta, B.R. and Dolly, J.O. (1997) Importance of two adjacent C-terminal sequences of SNAP-25 in exocytosis from intact and permeabilised chromaffin cells revealed by inhibition with botulinum neurotoxins A and E. *Biochemistry* **36,** 3061-3067.

10. Chen, F., Foran, P., Shone, C.C., Foster, K.A. and Dolly, J.O. (1997) Botulinum neurotoxin B inhibits insulin-stimulated glucose uptake into 3T3-L1 adipocytes and cleaves cellubrevin unlike type A toxin which failed to proteolyze the SNAP-23 present. *Biochemistry* **36,** 5719-5728.

11. Ashton, A.C. and Dolly, J.O. (1997) Microtubules and microfilaments participate in the inhibition of synaptosomal noradrenaline release by tetanus toxin. *J. Neurochem.* **68,** 649-658.

12. Dolly, J. O., Lawrence, G. W. and Foran, P. (1999) Distinct contributions to exocytosis of two adjacent C-terminal regions of SNAP-25 unveiled by botulinum toxins A and E. *Proc. of Biomedical Aspects of Clostridial Neurotoxins,* Oxford, ed. Tranter, H.S. p97-102.

13. O'Sullivan, G.A., Mohammed, N., Lawrence, G. W., Foran, P. G., Meunier, F. A., Ekong, T. A. N., Sesardic, D. and Dolly, J. O. (1999) SNAP(-25)ping out of botulinum neurotoxin A posioning: restoring regulated exocyosis in chromaffin cells. Proc. 10th Symposium on Chromaffin Cell Biology, p162.

14. Dolly, J. O., O'Sullivan, G. A., Mohammed, N., Foran, P. G., Lawrence, G. W., Lisk, G., Meunier, F. A. and dePaiva, A. (1999) Features influencing botulinum toxin-induced nerve sprouting and recovery from poisoning. *Movement Disorders.*

15. Dolly, J.O. (1998) Proc. C.N.R. Conference on Latrotoxin and Secretory Systems, p13.

16. Dolly, J.O. (1997) Proc. VI Internat. Symposium on Neurotoxins in Neurobiology, p29.

17. Dolly, J.O. (1997) J. Appl. Microbiol. **84**, S149.

18. Dolly, J.O. (1997) Proc. 2nd FEMS Internat. Meeting on Clostridia, p49.

Presentations at International Conferences

2000 **October,** Asilomar, California : Invited lecture on 'Molecular basis of the characteristic inhibitory effects of botulinum toxin serotypes on transmitter release' at the 37th annual meeting of the Interagency Botulism Research Coordinating Committee.

September, Chicago, Illinois : Executive speaker on 'Botulinum toxins : Overview of mechanisms of action and recovery' at the International Conference: Botulinum Toxin : Clinical, Immunological and Pharmacological Features.

September, Paris : Invited speaker on 'Botulinum neurotoxins as probes for exocytosis and synapse remodelling' at the 13th IST World Congress on Animal, Plant and Microbial Toxins.

April, Costa Brava, Spain : Guest presenter on 'Nerve regeneration after injury by toxins' at the European Science Foundation Conference on Mechanisms in Toxicity.

March, Cosenza, Italy : Overseas speaker on 'Neuro-exocytosis probed with botulinum toxins : nerve sprouting and remodelling as a means of cell survival' IV Workshop on Apoptosis in Biology and Medicine.

February, Alicante : Presentations on 'Ca^{2+} induces changes in the formation of SNAREs in chromaffin cells : evidence from altered susceptibility to trypsin' and 'SNAP-25 amino acid redundacy for regulated exocytosis - unveiled by rescue after poisoning chromaffin cells with botulinum neurotoxin A' at the III Winter School in Neurosciences.

1999 **November,** Orlando, Florida : Speaker on 'Rescue of exocytosis in botulinum toxin A-poisoned cells by tranfection and expression of toxin-resistant SNAP-25' at the 36th meeting of the Intragency Botulism Research Coordinating Committee.

November, Orlando, Florida : Invited keynote lecture on 'Features influencing botulinum toxin-induced nerve sprouting and recovery'at the International Conference on Basic and Therapeutic Aspects of Botulinum and Tetanus Toxins.

September, Fort Detrick : Lecture at USAMRIID on 'Molecular basis of the inhibition of exocytosis by botulinum toxin A : novel rescue therapy based on transfection of cells with toxin-resistant SNAP-25'.

July, Charring Cross Hospital, London : 'Neuro-exocytosis probed with botulinum toxins: basis for their clinical usefulness'

1998 **October**, Gaeta (LT), Italy : Keynote lecture on 'Botulinum toxins give insights into synapse remodelling and membrane trafficking' at the Latrotoxins and Secretory Systems Conference.

September, Sevilla, Spain : Invited lecture on 'Molecular basis of the therapeutic applications of botulinium toxin' at the 3rd Congress of the European Federation of Neurological Societies.

March, Scottsdale, Arizona : Invited keynote lecture on 'Protein engineering of botulinum toxin for research and therapeutic applications' at the Botulinum toxin: Science and Technology Conference.

1997 **December,** University of Reading, Reading : Organiser plus Keynote lecture on 'Exploitation of the multiple activities of botulinum and tetanus toxins' at the 'Neurochemical Group Colloquium - Exocytosis and Clostridial Toxins', organised and sponsored by The Biochemcial Society.

November, Nashville, Tennessee : Keynote address on 'Targeted intracellular therapy for botulinum toxins' at the AMSUS 104th Annual Meeting "Chemical, Biological and Radiation Threats: A Challenge for Federal Medicine" organised and sponsored by The Society of the Federal Health Agencies.

September, Parghelia, Italy : Invited keynote lecture on 'Therapeutic and research uses of botulinum toxin' at the VI International Symposium on Neurotoxins in Neurobiology, organised and sponsored by The Italian Society of Neuroscience.

July, Norwich, UK : Invited keynote lecture on 'Therapeutic and research uses of botulinum toxin' at the 'TOXINS' Conference, sponsored and organised by the Society for Applied Bacteriology.

June, Onzain, France : Invited lecture on 'Novel effects and applications of botulinum neurotoxins' at the Second International meeting on the Molecular Genetics and Pathogenesis of the Clostridia, sponsored and organised by the Federation of European Microbiological Societies

March, London : Invited platform lecture on 'Botulinum Toxins in the Future' at the 3rd European Botulinum Toxin Symposium, sponsored and organised by Allergan Inc.

Patent

Filed internationally in Aug.1999 entitled 'Methods of Treatment'

Degrees obtained

Drs N. Mohammed and G. O'Sullivan awarded Ph.Ds.

CONCLUSIONS

In summary, the following points should be emphasised. First, in neurons and neuroendocrine cells BoNT/A-LC protease remains active for at least three weeks post-intoxication. Second, BoNT/A

poisoning in neuro-endocrine chromaffin cells can be rescued through the expression of BoNT/A-resistant SNAP-25. These novel findings establish the proof of principle for fast rescue from BoNT/A intoxication by this innovative but straight forward transfection process. Finally, for the effective application of this rescue strategy first to neurons in culture, prior to botulinised neurons *in situ,* we have successfully adopted 'state of the art' safe, replication-incompetent, adenoviral technology in this laboratory for highly efficient neuronal transfection and proven its effectiveness. A recent application for funding to develop this promising therapy for clinical use has been made to USAMRMC entitled 'Therapies for arrest and rescue from botulism: cholinergic targeted-delivery, via an atoxic BoNT/E vehicle, of inhibitors of botulinum toxins and SNAREs non-cleavable by their proteases'.

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APPENDICES

TABLE 1.

Comparison of the kinetic parameters for BoNT/A cleavage of full-sized SNAP-25, 26- and 17-mer peptides

Substrate	Km (mM)	Vmax (μmoles min^{-1} mg^{-1}	k_{cat} (sec^{-1})	v_o at 15μM (μmoles min^{-1} mg^{-1})	Turnover rate at 15 μM (sec^{-1})
17-mer (NH-187-203CA)*	0.63	3.1	7.7	0.16	0.40
26-mer (residues 181-206)*	0.72	0.5	1.3	0.02	0.06
Full-length@ SNAP-25	>> 0.015	?	?	12.6	31.5

* Assayed using RP-HPLC or ELISA @Incubations with peptide substrates were at 37°C using 10 nM-reduced BoNT/A in 35 mM HEPES pH 7.5 containing 2 mM DTT, 0.8 $mgml^{-1}$ BSA and 100 μM $ZnCl_2$. Alternatively, toxin incubations with full-length GST-SNAP-25 were performed as detailed in the Materials and Methods. Km and Vmax values were obtained from Lineweaver-Burke plots.

TABLE 2.

The overall susceptibilities of wild-type and various SNAP-25 mutants to cleavage by BoNT/A

SNAP-25 variant	**Position of mutated residue(s)**	**Minimum BoNT/A concentration (nM) necessary to proteolyse 50% of SNAP-25 in the standard assay**[a]	**Susceptibilities to proteolysis by BoNT/A (relative to wild-type)**[b]
Wild-type	None	0.0045 (0.022)	1.0
Q197/R198			
Q197A	P′1	0.0054	1.2
R198A	P′1	2.5	560
R198T	P′1	72	16000
A199L	P′2	(1.09)	49
Q197A/R198A	P1, P′1	20	4400
Q197A/R198K	P1, P′1	170	38000
Q197A/R198E	P1, P′1	170	38000
Q197K/R198H	P1, P′1	2.0	440
Q197W/R198W	P1, P′1	0.43	96
Q198A/R199K/ A199L	P2, P′1, P′2	>>100 nM	>>3000
N196Q/Q197A/ R198K	P1, P′1, P′2	>>100 nM	>>3000
SNAP-$25_{1\text{-}202}$	Removal of 4 residues from C-terminus	(0.031)	1.4
SNAP-$25_{ADD\ 1\text{-}206\ (+18)}$	Addition of 18 residues to C-terminus	(0.16)	7.3

[a] Standard curves, relating increasing amounts of intact GST-SNAP-25 per well with the increasing A405 nm values recorded using either anti-SNAP-25-(C-terminus)-Ig or, anti-SNAP-25_{A1}-pAb, were used to calculate the A405nm reading which was equivalent to 50 % intact SNAP-25 remaining in wells. These absorbance values were used to extrapolate the concentrations of BoNT/A required to proteolyse 50 % of either the wild-type or mutant SNAP-25 polypeptides from Figure 11. Whilst incubations with toxin lasted 6 h in experiments shown in Figure 11A,B, in part C only 90 min incubations were performed; thus, in this case the toxin concentrations necessary for 50 % cleavage of the mutants compared to wt are indicated inside brackets.

[b] Values obtained by dividing the minimal BoNT/A concentration giving 50% cleavage of the SNAP-25 mutant being tested by the minimal concentration which cleaves wild-type; larger values indicate greater resistance to proteolysis.

FIGURE LEGENDS

Figure 1. Expression and affinity purification of GST-linked SNAP-25$_{FL}$ and SNAP-25$_A$: analysis by SDS-PAGE and Western blotting. Cultures of bacteria containing the pGEX-2T plasmid harboring the SNAP-25 gene for the entire amino acid sequence (residues 1-206; panels A, B) or a fragment (residues 1-197; panels C,D) were induced with IPTG for 3 h at 37°C before being collected by centrifugation. Bacteria were lysed by brief sonication and lysozyme treatment; the supernatants resulting from ultracentrifugation of the latter homogenates (lanes 1) were added to columns containing glutathione-Sepharose. After multiple passages, the breakthroughs were collected (lanes 2) and unbound protein washed away. GST-linked proteins were eluted biospecifically using glutathione (peak fraction are shown in lanes 3 and 4). Samples were subjected to SDS-PAGE and proteins detected by Coomassie staining (panels A and C) or electrophoretically transferred to Immobilon-P membranes and immunoblotted with anti-SNAP-25 (C-terminus) (panel B) or anti-SNAP-25$_A$ (panel D) selective Ig. Primary Ig binding was assessed using anti-species specific antibodies conjugated with alkaline phosphatase and the latter were visualised colourimetrically using BCIP and NBT substrates. Lane 5 shows wide-range molecular weight protein standards of the sizes indicated.

Figure 2. BoNT/A proteolysis of recombinant GST-SNAP-25 assessed using immunoblotting. GST-SNAP-25 (0.1 μM) was incubated in the absence or presence of the specified concentrations of pre-reduced BoNT/A at 37°C for 30 min in KGEP buffer (see Methods) supplemented with 0.2 mg/ml BSA, 1 mM DTT and 50 μM $ZnCl_2$. Equal amounts of the resultant samples were subjected to immunoblotting using the primary antibodies specified. Primary Ig binding was detected using anti-species antibodies conjugated with horseradish peroxidase and the latter were visualised using ECL (detailed in Methods).

Figure 3. Dot-blot analyses of the selectivities of affinity-purified anti-SNAP-25$_A$-Ig preparations for either full-length or BoNT/A-truncated SNAP-25. Immobilon-P strips dotted with the indicated amounts of either GST-linked full-length or SNAP-25$_A$ proteins were incubated overnight at 4°C with the specified quantities of purified IgGs or dilutions of antiserum. Primary Ig binding was visualised as outlined in the legend to Figure 1 and development times were identical for all strips.

Figure 4. Proteolysis by BoNT/A of GST-SNAP-25 coated onto polystyrene wells detected using Igs selective for the intact substrate or the toxin cleavage product: reduction of the interchain disulphide is essential. Wells of ELISA plates pre-coated with equal amounts of GST-SNAP-25

were exposed in the absence or presence of increasing concentrations of either pre-reduced (exposure at 37°C to 20 mM DTT for 60 min; filled symbols) or non-reduced BoNT/A (open symbols) for 2 h at 37°C in KGEP buffer (supplemented as specified in the legend to Figure 2). Following washing of the wells, 75 µl of primary Igs at 1.0 µg/ml in blocking buffer were added and incubated for 16 h at 4°C Primary Igs [anti-SNAP-25_{A1} Ig (A) or anti-SNAP-25 Ig (B)]retained in wells were detected using a 2 h incubation at 23°C with anti-species-specific Igs conjugated with alkaline phosphatase (used at 1:1000 dilutions and 100 µl/well). Following removal of secondary antibodies by washing, 100 µl of an alkaline buffer containing 3.5 mM pNPP substrate was added and the colour development at 405 nm which followed measured in an ELISA plate reader. Absorbance values recorded after the same development period (following subtraction of the basal A405 nm value in wells lacking a SNAP-25 coat but treated identically with primary and secondary Igs) are plotted; data are representative of means (± S.D.; n = 4).

Figure 5. Standard curves relating amounts of GST-SNAP-25_A or GST-SNAP-25_{1-206} per well to the A405 nm in ELISA performed using anti-SNAP-25_{A1} or anti-SNAP-25 (C terminus) specific Igs. The wells of ELISA plates were coated for 90 min at 23°C with 50 µl of 2 µg/ml mixtures of GST-SNAP-25_A and GST-SNAP-25_{1-206} proteins (with different relative percentages of each in panels A, B, and C). In D and E, wells were coated with slightly different mixtures of GST-SNAP-25 and GST-SNAP-25_A. All were incubated for 16 h at 4°C with anti-SNAP-25_{A1}-Ig (A to C at 2.7 µg Ig/ml) or anti-SNAP-25 (C terminus)-Ig (D and E at 1.2 µg Ig/ml). Primary Ig binding was detected exactly as outlined in the legend to Figure 4. The A405 nm values expressed are means (± S.D.; n = 6 or 8) with the values for 0-10% GST SNAP-25_A range presented in an expanded form in part B. In panels C and E, absorbance values after 6 and 3 minute development, respectively using anti-SNAP-25_{A1}-Ig or anti-SNAP-25_{FL}-Ig, were expressed as percentages relative to the maximum values recorded (therefore, termed: Δ A405 nm).

Figure 6. Lineweaver-Burke plot of initial rates of proteolysis by reduced BoNT/A for various concentrations of GST-SNAP-25.

A modified ELISA was employed to determine the initial rates of BoNT/A cleavage of GST-SNAP-25 at concentrations between 0.118 to 15 µM. (see Material and Methods). Each data point plotted are means of 16 determinations (means ± S.D).

Figure 7. A surrogate peptide encompassing the S4 SNARE motif inhibits BoNT/A cleavage of GST-SNAP-25, 26- and 17-mer peptide substrates. A diagrammatic representation of the SNAP-25 molecule is shown indicating important regions. A S4-motif peptide of the sequence specified was pre-incubated for 60 min at 37°C with reduced BoNT/A prior to addition to the specified substrates lacking this S4 region which are poorly proteolysed by BoNT/A. Initial rates of cleavage by BoNT/A of the 17- and 26-mer substrates (50 μM) or GST-SNAP-25 (0.5 μM) performed at 37°C, in the absence or presence of various concentrations of S4-motif peptide, were assessed by an HPLC assay (analogous to that used by Schmidt and Bostain, 1995, 1997) and ELISA, respectively. The concentrations of the S4-motif peptide necessary to inhibit the initial cleavage rates by 50% for the different substrates are displayed.

Figure 8. Diagrammatic representation of the mutations, deletions and additions to SNAP-25. Schematic of the mutations of the SNAP-25 protein generated as GST fusions and code names [i.e. R198T indicating that arginine at position 198 in the amino acid sequence (shown using the single letter code) is changed to threonine].

Figure 9. Assessment of the purity and immuno-reactivities of the affinity-purified SNAP-25 mutants by SDS-PAGE and/or Western blotting. GST-fusion proteins were isolated form IPTG-induced bacterial cultures by affinity chromatography. The biospecifically-eluted proteins were subjected to SDS-PAGE and visualised by Coomassie staining (A) or transferred to Immobilon-P membranes and detected (B and C) using two different antibodies. In B, an antibody raised against a SNAP-25 peptide (residues 190-197; termed anti-SNAP-$25_{190-197}$-Ig) which reacts equally well with the full-length and BoNT/A-truncated SNAP-25 was used. In C, anti-SNAP-25 (C-terminus)-Ig was employed. Primary Ig binding was detected using anti-species Ig conjugated with alkaline phosphatase and visualised colourimetrically.

Figure 10. Western blotting of affinity-purified GST-linked addition and deletion mutants of SNAP-25. In A, the relative sizes of the expressed GST-SNAP-25 fragments deleted at the N or C-terminus or with additions to the C-terminus are indicated diagrammatically. Equal amounts (50ng) of the specified fusion proteins, purified as outlined in the legend to Figure 1, were subjected to immunoblotting using the anti-SNAP-$25_{190-197}$-Ig (detailed in the legend to Figure 9). Primary Ig binding was visualised indirectly using alkaline phosphatase conjugated anti-guinea-pig antibodies and developed as specified in the legend to Figure 1.

Figure 11. Assessment by ELISA using two different anti-SNAP-25-specific Igs of the susceptibilities of wt or mutant GST-linked SNAP-25s to proteolysis by BoNT/A. ELISA plate wells were coated with either wild-type or the specified single (A), double (B) mutants and (C) addition or deletion mutants of SNAP-25 before exposure for 6 h (A and B) or (C) 90 min at 37°C to DTT-reduced BoNT/A. The amounts of wild-type or mutant SNAP-25s remaining were probed with either (A and B) anti-SNAP-25-(Cterminus)-Ig for intact SNAP-25 or (C) the truncated product using anti-$SNAP\text{-}25_A$-pAb. Binding of these latter antibodies was quantified using alkaline phosphatase-conjugated anti-species antibodies and a colorimetric assay. Absorbance values at 405 nm are expressed either as percentages of the maximal values recorded for SNAP-25-containing/toxin-free control wells (A and B) or in arbitrary units. Data are means (± S.D.; n = 4-6).

Figure 12. Assessment of BoNT/A cleavage of wild-type and triple-point mutants of SNAP-25 using SDS-PAGE and protein staining. Reduced BoNT/A at the specified concentrations was incubated with either 0.5 μM wt GST-SNAP-25 (A), NQRA; N196Q/Q197A/R198K (B), QAKA; Q197A/R198K/A199L mutant (C) fusion proteins for 2 h at 37°C. Samples were fractionated by SDS-PAGE on 8% acrylamide gels, fixed and proteins visualised by a silver staining method.

Figure 13. Monitoring the protracted BoNT/A- and /B-induced inhibition of catecholamine release in chromaffin cells and assessment of their SNAP-25 and Sbr/Cbr contents. Intact chromaffin cells were incubated at 37°C in LISM for 24 h in the absence (open bars) or presence (hatched bars) of 6.6 nM BoNT/A (A) or 66 nM BoNT/B (B). After replacement of LISM with the normal medium (Lawrence et al., 1996), the cells were maintained in culture for up to 56 days with weekly substitution of the medium. At the specified times, Ba^{2+} -evoked catecholamine release over 15 min was quantified fluorometrically by subtraction of basal release values, measured in Locke's buffer from that induced by 2 mM Ba^{2+} in the same buffer. The resultant values were expressed as a percentage of the total cell content of catecholamines. Evoked exocytosis was then calculated by expressing the % catecholamine release of toxin-treated cells as a percentage of toxin-free controls (means ± S.E.M; n = 4). Immediately after measuring secretion, membrane fractions were prepared, solubilised in SDS, boiled for 5 min and subjected to SDS-PAGE using 12.5% gels, followed by immunoblotting with the indicated antibodies.

Figure 14. Expression of wild-type and SNAP-25 mutants in transfected CHO cells. CHO cells, that lack SNAP-25, were transfected with the pcDNA1.1/Amp vector incorporating the specified SNAP-25 gene using the Superfect™ reagent. The transfected cells were treated with LISM in the

absence or presence of 6.6 nM BoNT/A (indicated). Two days later, the membrane fraction was isolated from the cells and equal amounts of SDS solubilised proteins were subjected to immunoblotting, using the indicated antibodies. Chromaffin cell membrane fraction was run as a control. The relative amounts of primary antibodies bound were visualised by ECL and only the relevant track positions are shown.

Figure 15. Expression of protease-resistant SNAP-25 mutants, but not wild-type, rescues evoked secretion from BoNT/A-pre-treated cells: sole monitoring of transfected cells using the hGH-based reporter system. (A) Intact chromaffin cells were treated for 24 h with LISM, in the absence (open bars) or presence (hatched bars) of 6.6 nM BoNT/A, as described in the legend to Figure 13. After a 24 h recovery period, the resultant cells were transfected with the pcDNA1.1/Amp mammalian expression vector containing the specified SNAP-25 or CAT gene together with the reporter plasmid encoding human growth hormone (hGH; as outlined in the Materials and Methods). After five days, the hGH secretion only occurring from co-transfected cells under basal and stimulated conditions (using buffers omitting or containing Ba^{2+}), were quantified using a radio-immunoassay (means ± S.E.; n=4). The evoked component of secretion was expressed as a percentage of the measured total cell content. (B) Cells pre-treated with LISM in the absence (open bars) or presence (hatched bars) of 6.6 nM BoNT/A were subsequently transfected with vectors encoding hGH with or without the plasmid encoding the BoNT/A protease-resistant SNAP-25 R198T mutant (indicated), and cultured for an additional five days. These cells were then permeabilised using a digitonin-containing permeabilisation buffer excluding or containing (black bar) 100 nM of DTT-reduced BoNT/E for 15 min. The Ca^{2+}-evoked component of hGH secretion, measured over the subsequent 15 min period, was quantified (means ± S.E.; n=4) and expressed as a percentage of the total cell content.

Figure 16. BoNT/A proteolytic activity persists in poisoned cells for at least 3 weeks; only protease-resistant SNAP-25 mutants rescue evoked secretion. Intact chromaffin cells were treated for 24 h with LISM, in the absence (open bars) or presence (hatched bars) of 6.6 nM BoNT/A, as described in the legend to Figure 13. After 16 days, the resultant cells were transfected with the pcDNA1.1/Amp mammalian expression vector containing the specified SNAP-25 or CAT gene together with the reporter plasmid encoding hGH, as outlined in the Materials and Methods section. After a a further five days, Ba^{2+}-evoked hGH secretion was assessed (as outlined in the legend to Figure 13).

Figure 17. Truncation and mutation of SNAP-25 to determine the C-terminal residues required for exocytosis. (A) Intact chromaffin cells were treated for 24 h with LISM in the absence (open and filled bars) or presence (hatched bars) of 6.6 nM BoNT/A, as described in the legend to Figure 13. The cells were then transfected with the pcDNA1.1/Amp expression vector containing either the indicated BoNT/A-resistant SNAP-25 (see panel B; open and hatched bars) or wild-type (filled bars) construct of specified length together with the reporter plasmid encoding hGH, as outlined before. After five days, Ba^{2+}-evoked hGH secretion was assessed (as outlined in the legend to Figure 13). This experiment was repeated twice, with the pattern being the same in each case. (B) The C-terminal amino acid sequence of SNAP-25 is shown, incorporating wild-type R^{198}, or T^{198} as well any other mutations in bold letters; deletions are specified by residue numbers. Also shown is the helical wheel representation of the tetrameric SNARE complex, with the heptad positions labelled from 'a' through 'g', looking down the centre of the helix from the top at Q^{197} (arrow) and ending at L^{203}, within a representative C-terminal repeat of SNAP-25 (Poirier *et al.*, 1998).

Figure 18. Blockade of neuroexocytosis in cerebellar neurons by BoNT/A lasts for many weeks co-incident with proteolysis of SNAP-25: its protease persists for at least 2 weeks as revealed using [^{35}S]-Met-labeling and immunoprecipitation of newly-synthesized SNAP-25. Cerebellar neurons, maintained for 7 days in culture, were exposed for 24 h in the absence or presence of 5 pM BoNT/A and maintained by regular changes of medium. A, at the specified post-intoxication periods, the abilities of control and toxin-treated neurons to exhibit neuroexocytosis was determined as outlined in Methods, and results expressed relative to the maximal proportion of Ca^{2+}-dep K^+-evoked exocytosis (~ 65% of total) found to be inhibitable at each interval with 200 pM BoNT/A. B, the above samples were subjected to immunoblotting and intact or truncated SNAP-25 were detected using their respective IgGs. C, control or BoNT/A pre-poisoned neurons were radio-labelled using a 4h 'pulse' with [^{35}S]-Met containing medium, immediately washed and detergent solubilised (0 h chase), or cultured in the absence of labelling medium for a 14 h chase, prior to harvesting. The newly-synthesised SNAP-25 was isolated via immunoprecipitation using SMI-81 mAb on anti-mouse IgG agarose prior to autoradiography or immunoblotting.

Figure 19. BoNT/E causes a short-lived appearance of SNAP-25$_E$ and inhibition of transmitter release from cerebellar neurons : co-poisoning with BoNT/E and /A fails to shorten the prolonged blockade of exocytosis caused by the latter and the predominance of SNAP-25$_A$. Neurons maintained for 8 days *in vitro* were exposed for 24 h in the absence or presence of BoNT/A, BoNT/E or

both neurotoxins (as specified), and maintained by regular changes of medium. At the specified post-intoxication periods, the abilities of control and toxin-treated neurons to exhibit neuroexocytosis was determined as outlined in Methods. The resultant values are expressed as % of the total transmitter content and, in parenthesis, as % inhibition of this component relative to the appropriate control. The above samples were subjected to immunoblotting and intact or truncated SNAP-25 was detected using ECL and SMI-81 mAb, as detailed in Methods.

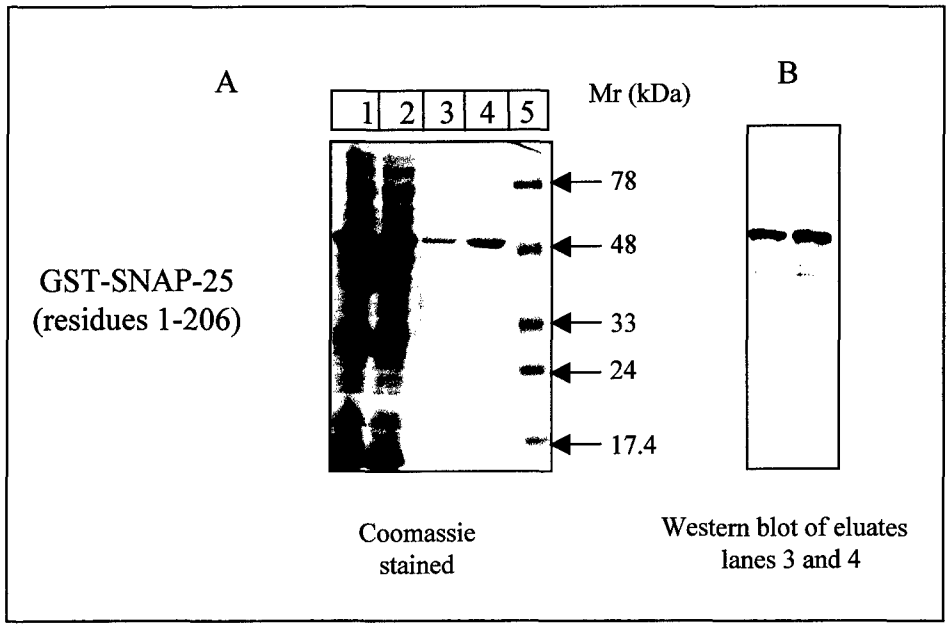
Figure 20. Adenoviral infection of cerebellar granule neurons in culture detected using a β-galactosidase marker to visualise expression. Neurons (~ 1 x 10^6) prepared as outlined in the legend to Figure 18 and maintained in culture for 14 days were incubated for an additional 3 days in the absence (A) or presence (B) of a replication-deficient adenovirus [~ 3 x 10^8 p.f.u; referred to as Ad RSV β-gal (Schneider *et al.,* 2000)] that expresses a nuclear-localising β-galactosidase (β-gal) marker. Both samples were fixed and stained for β-gal enzyme reactivity. A selected low-magnification field is shown; note that almost all neurons in panel B are infected.

Figure 21. Subcloning strategy to produce recombinant adenovirus. (A) The cDNAs encoding mouse wild-type SNAP-25b, SNAP-25, Q198T, $SNAP\text{-}25_A$, $SNAP\text{-}25_E$ and GFP were digested with *Bam*H1 and *Not*1 prior to gel purification. (B) All genes were cloned uni-directionally into pre-digested pTRE-Shuttle vector (shown diagrammatically) and ligation of the appropriate genes was confirmed by restriction digest analysis of positive transformants. (C). Positive recombinants were exposed to two unique enzymes (PI-*Sce* 1 and I-*Ceu* 1) to excise the cDNA inserts as well as the TRE-CMV promoter from the Shuttle vector; the integrity of the cassettes was confirmed by agarose gel electrophoresis (C). Cassettes were then ligated uni-directional into a pre-digested Adeno-X viral DNA construct (shown diagrammatically, D); recombinants were screened by PCR, sequencing and restriction digest mapping. Viral DNA was linearised with Pac 1 before infecting HEK 293 (E) cells which were grown for up to 2-3 weeks to obtain high viral titres.

Figure 22. Adenoviral infection of rat dorsal root ganglionic neurons in culture using the Adeno-X system revealed by expression of the Green Fluorescence Protein (GFP). Peripheral neurons (~ 1 x 10^5/ well) were dissociated from 125-150g rats and maintained as outlined in Winter *et al.* (1988). Cells were infected after 24h in culture with 1 x 10^7p.f.u each of Adeno-X-Tet-Off regulatory and Adeno-X TRE-GFP viruses. After 3 days in culture, unfixed neurons were visualized by phase contrast (A) and fluorescent microscopy for GFP-expressing infected neurons (B). A selected low magnification field is shown; note, not all neurons are fluorescently labeled by this procedure.

FIGURES 1 - 22

Figure 1


A
B
1 2 3 4 5
Mr (kDa)
78
48
33
24
17.4
GST-SNAP-25
(residues 1-206)
Coomassie
stained
Western blot of eluates
lanes 3 and 4

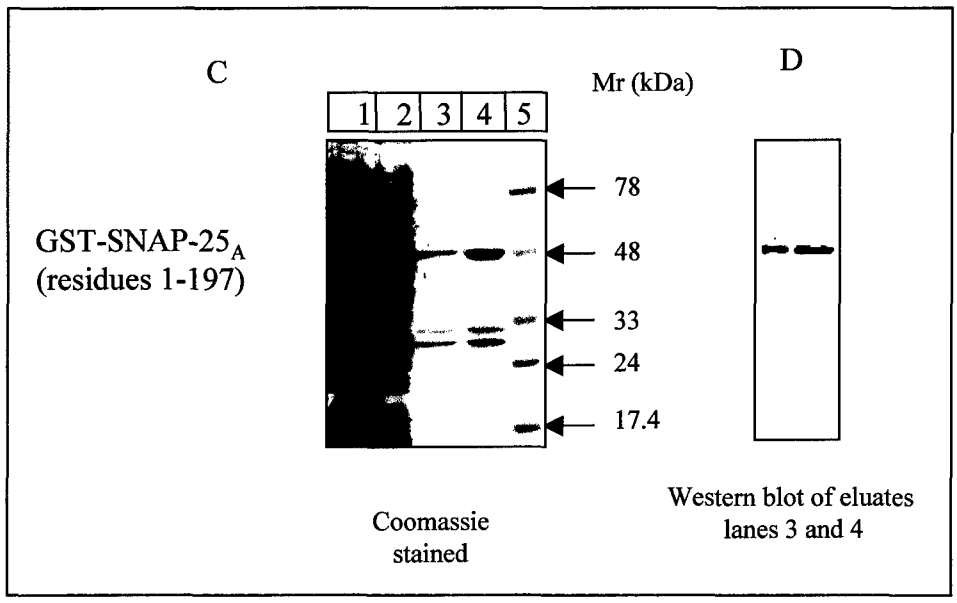
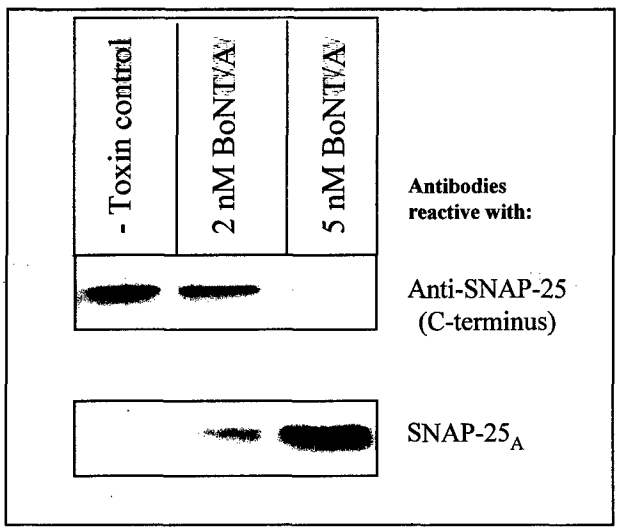

C
D
1 2 3 4 5
Mr (kDa)
78
48
33
24
17.4
GST-SNAP-25A
(residues 1-197)
Coomassie
stained
Western blot of eluates
lanes 3 and 4

Figure 2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5 nM BoNT/A
Antibodies reactive with:
Anti-SNAP-25 (C-terminus)
SNAP-25A

Figure 3

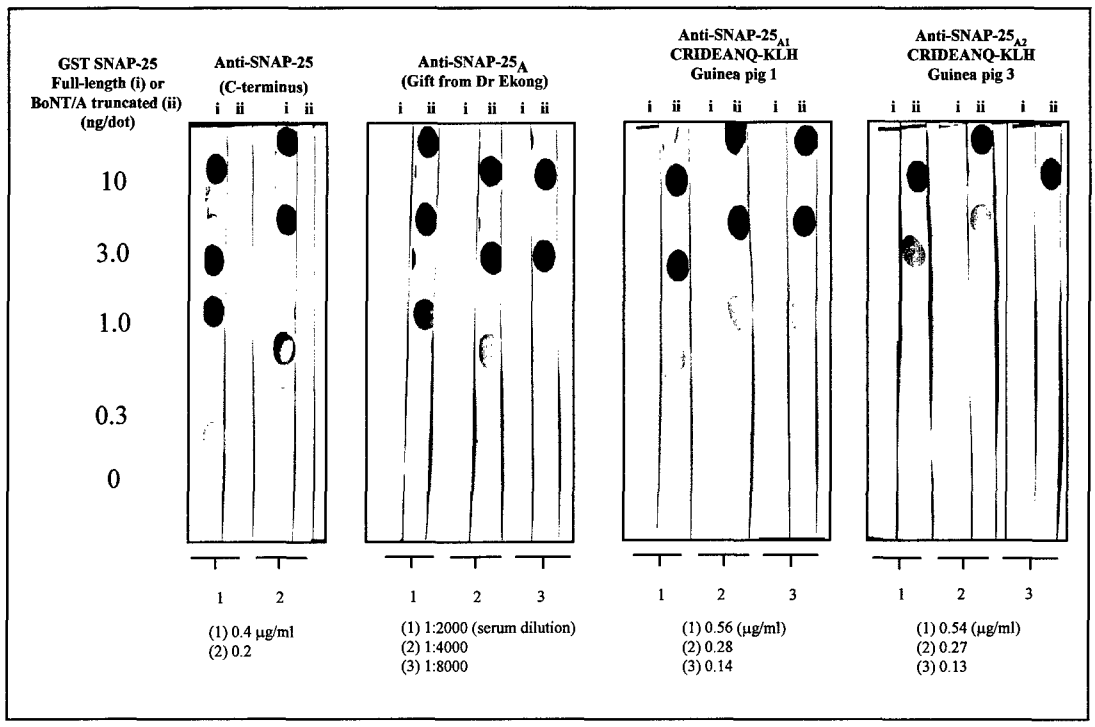
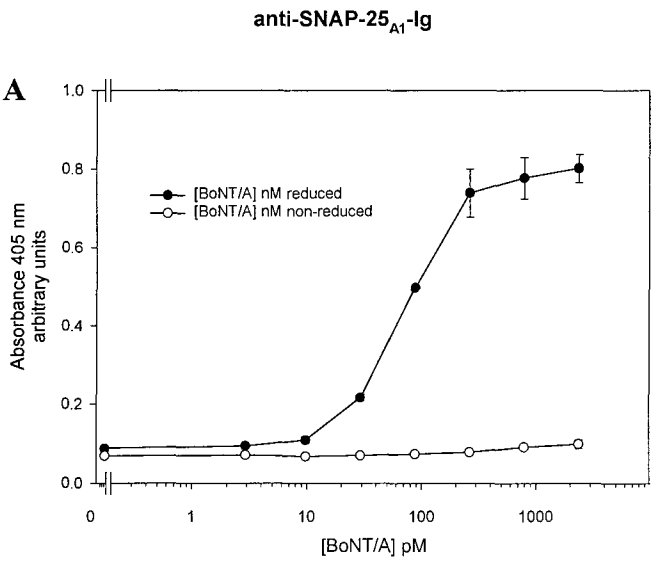

GST SNAP-25
Full-length (i) or
BoNT/A truncated (ii)
(ng/dot)
10
3.0
1.0
0.3
0
Anti-SNAP-25
(C-terminus)
i ii i ii
1 2
(1) 0.4 µg/ml
(2) 0.2
Anti-SNAP-25A
(Gift from Dr Ekong)
i ii i ii i ii
1 2 3
(1) 1:2000 (serum dilution)
(2) 1:4000
(3) 1:8000
Anti-SNAP-25A1
CRIDEANQ-KLH
Guinea pig 1
i ii i ii i ii
1 2 3
(1) 0.56 (µg/ml)
(2) 0.28
(3) 0.14
Anti-SNAP-25A2
CRIDEANQ-KLH
Guinea pig 3
i ii i ii i ii
1 2 3
(1) 0.54 (µg/ml)
(2) 0.27
(3) 0.13

Figure 4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A
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0.8
0.6
0.4
0.2
0.0
Absorbance 405 nm arbitrary units
[BoNT/A] nM reduced
[BoNT/A] nM non-reduced
0
1
10
100
1000
[BoNT/A] pM

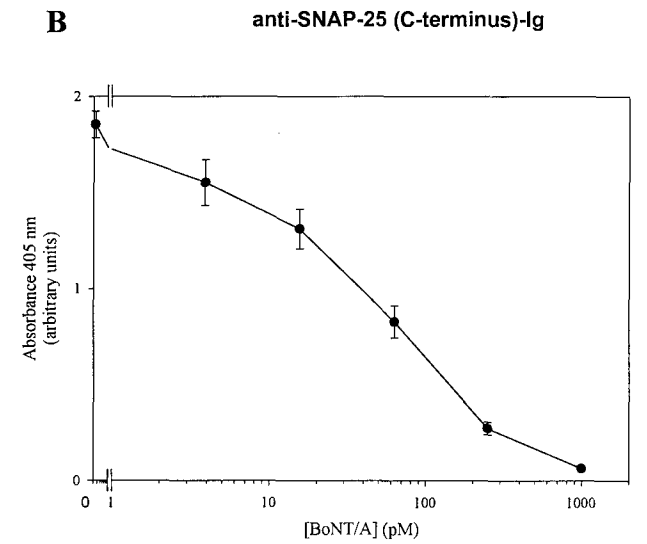

B
anti-SNAP-25 (C-terminus)-Ig
2
1
0
Absorbance 405 nm (arbitrary units)
0
1
10
100
1000
[BoNT/A] (pM)

Figure 5

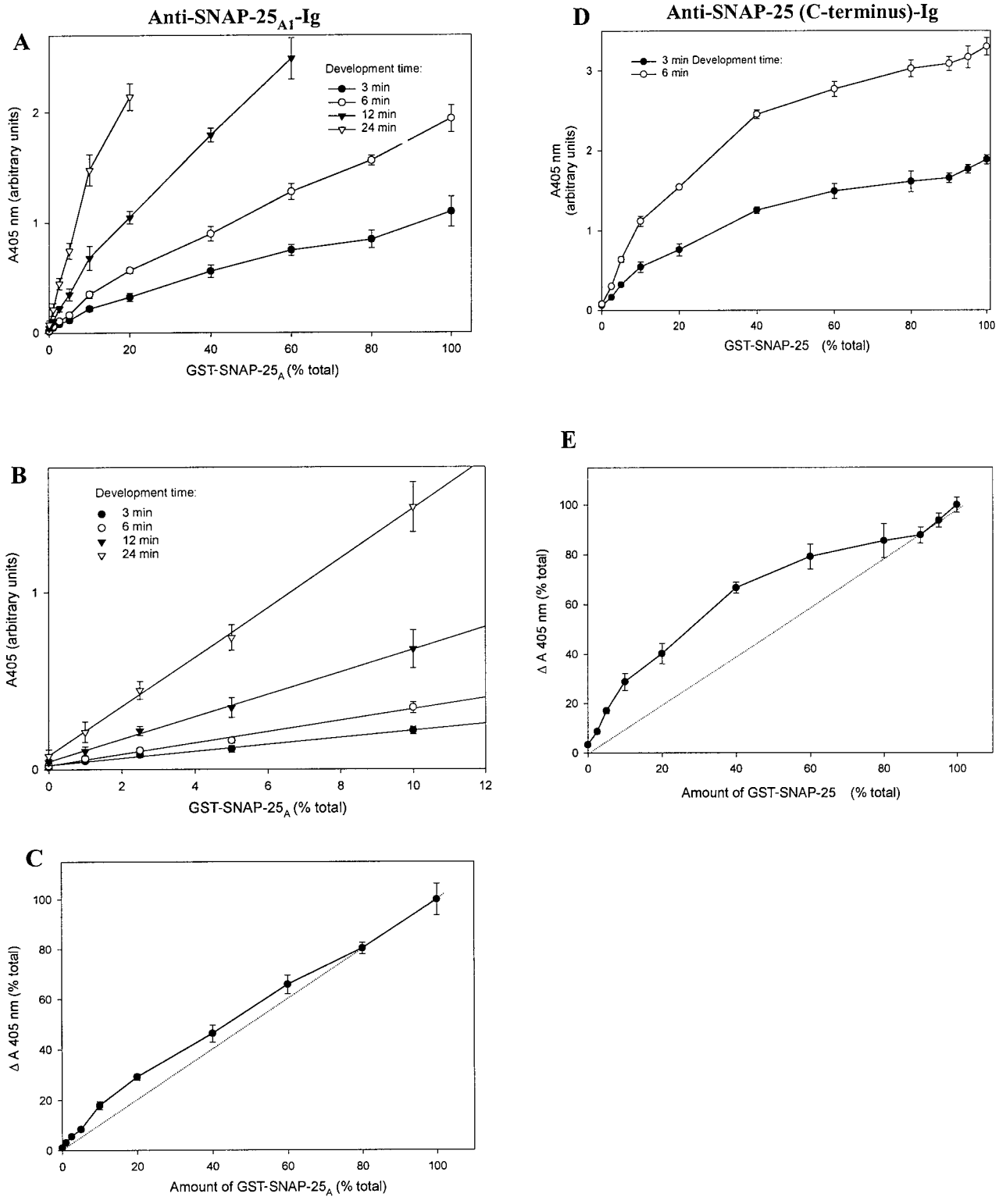
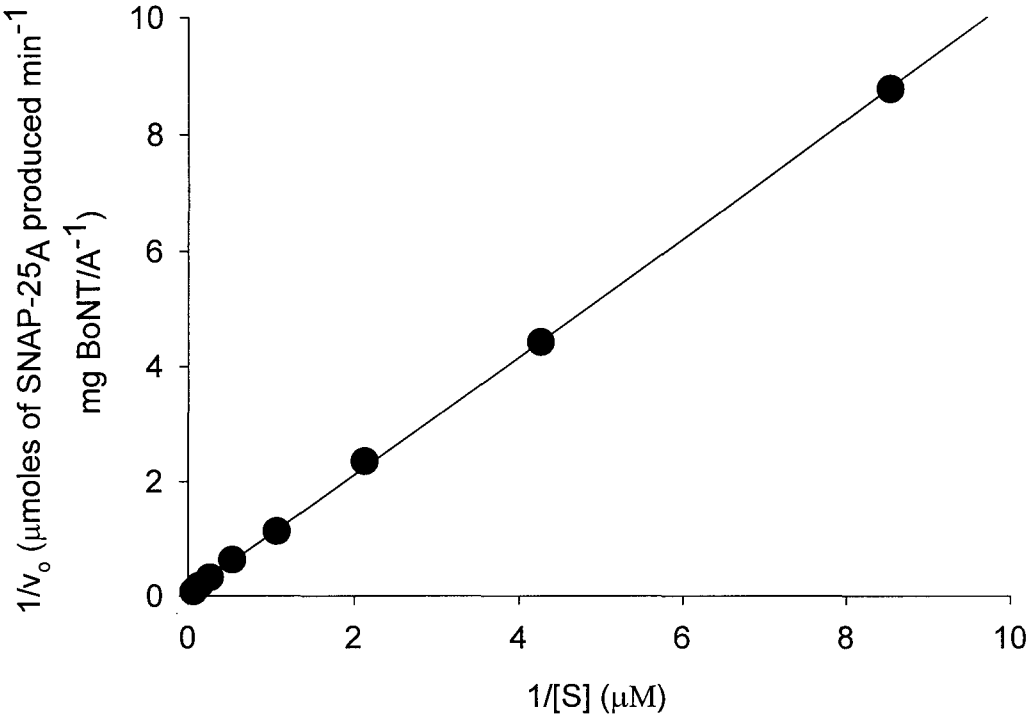
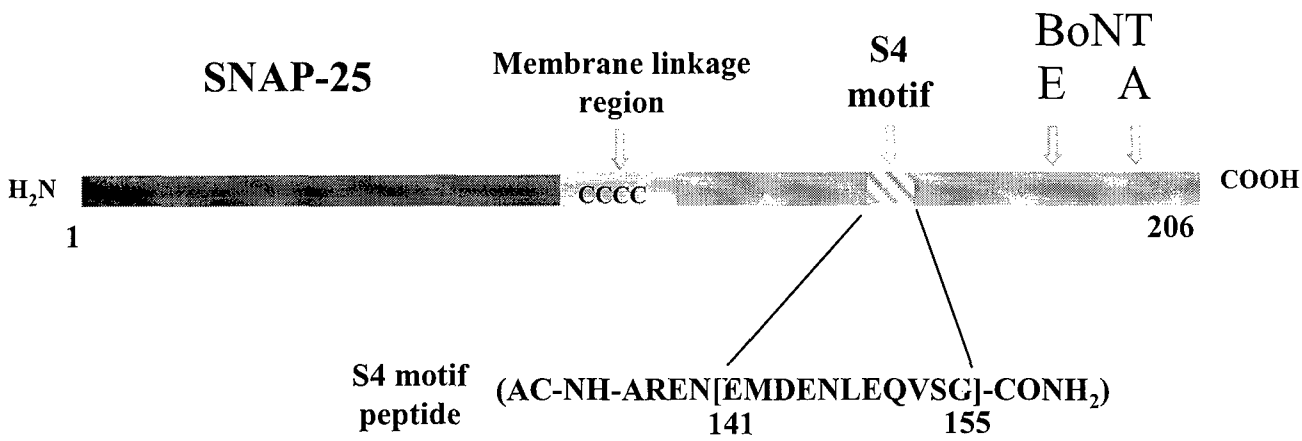

A
Anti-SNAP-25A1-Ig
Development time:
3 min
6 min
12 min
24 min
A405 nm (arbitrary units)
GST-SNAP-25A (% total)
D
Anti-SNAP-25 (C-terminus)-Ig
3 min Development time:
6 min
A405 nm (arbitrary units)
GST-SNAP-25 (% total)
B
Development time:
3 min
6 min
12 min
24 min
A405 (arbitrary units)
GST-SNAP-25A (% total)
E
Δ A 405 nm (% total)
Amount of GST-SNAP-25 (% total)
C
Δ A 405 nm (% total)
Amount of GST-SNAP-25A (% total)

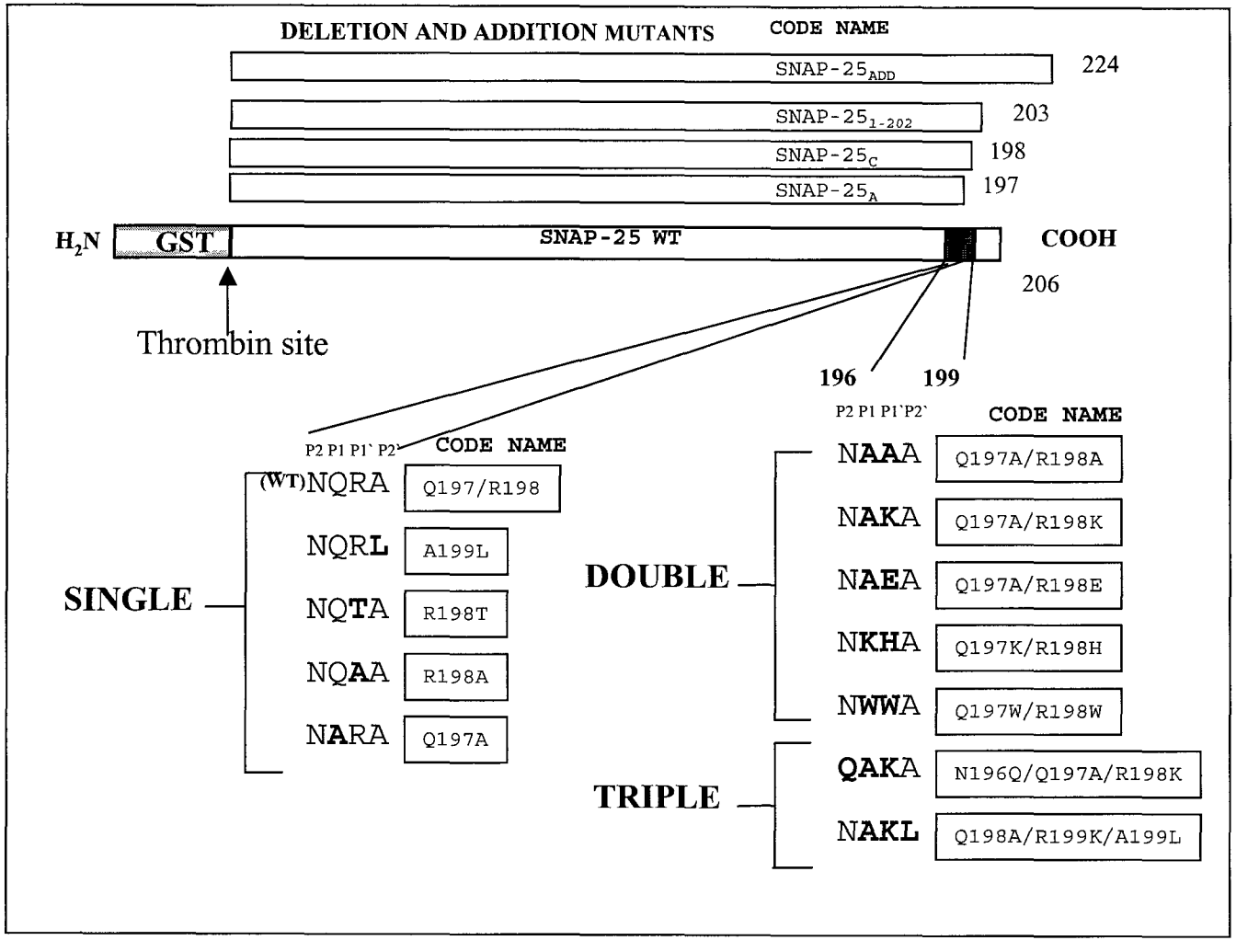
Figure 6


1/v_o (μmoles of SNAP-25$_A$ produced min^{-1} mg BoNT/A^{-1})
10
8
6
4
2
0
0
2
4
6
8
10
1/[S] (μM)



IC_{50} (mM)		
~ 2	17-mer [NH-187-203CA]	Ac- -$CONH_2$ (187 – 203)
~ 2	26-mer substrate	H_2N-Cys- -COOH (181 – 206)
~ 0.5	Full-length SNAP-25	

Figure 8


DELETION AND ADDITION MUTANTS
CODE NAME
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SNAP-251-202
203
SNAP-25C
198
SNAP-25A
197
H2N
GST
SNAP-25 WT
COOH
206
Thrombin site
196
199
P2 P1 P1` P2`
CODE NAME
(WT)NQRA
Q197/R198
NQRL
A199L
NQTA
R198T
NQAA
R198A
NARA
Q197A
SINGLE
DOUBLE
P2 P1 P1`P2`
CODE NAME
NAAA
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NAKA
Q197A/R198K
NAEA
Q197A/R198E
NKHA
Q197K/R198H
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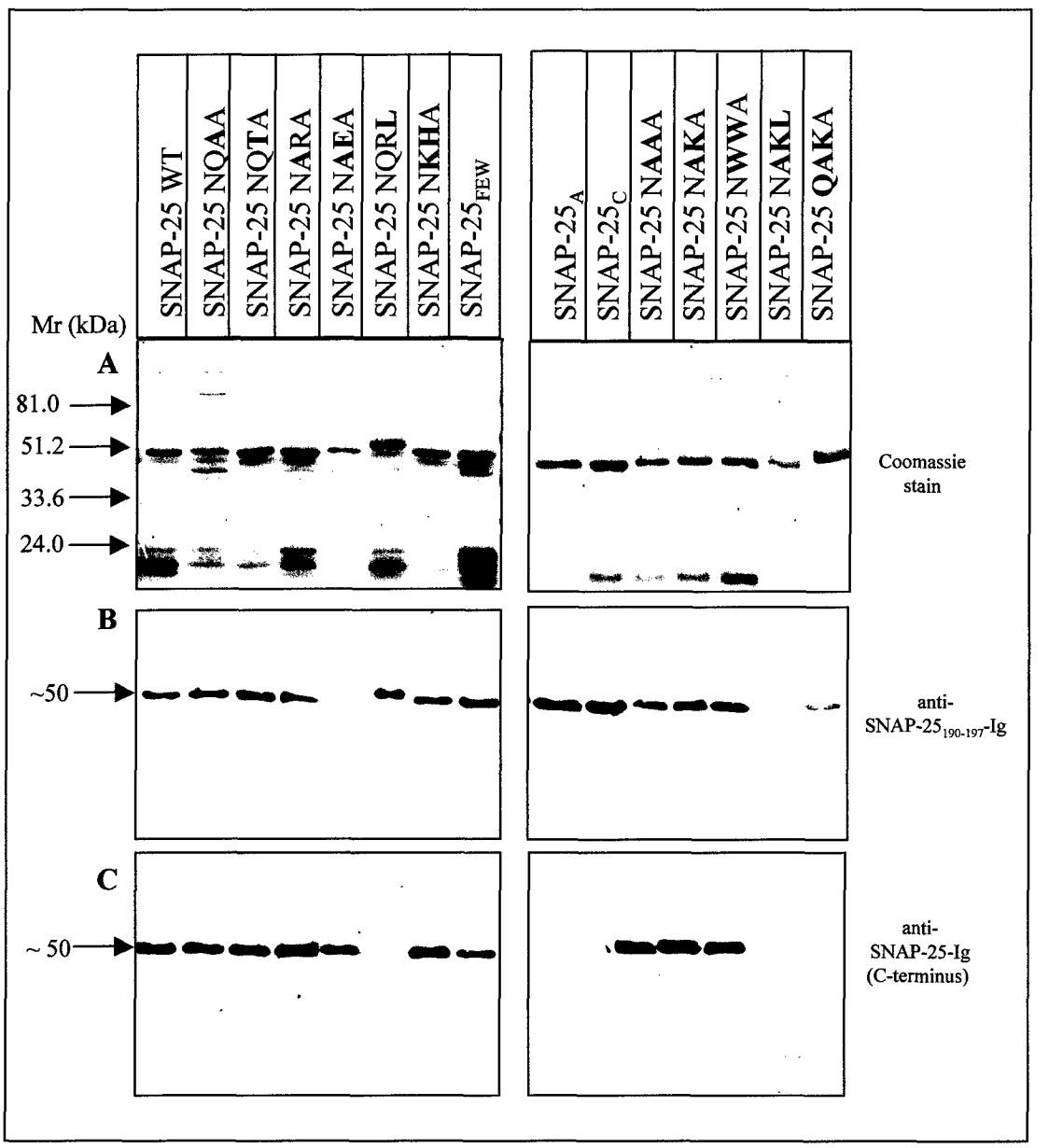
NAKL
Q198A/R199K/A199L

Figure 9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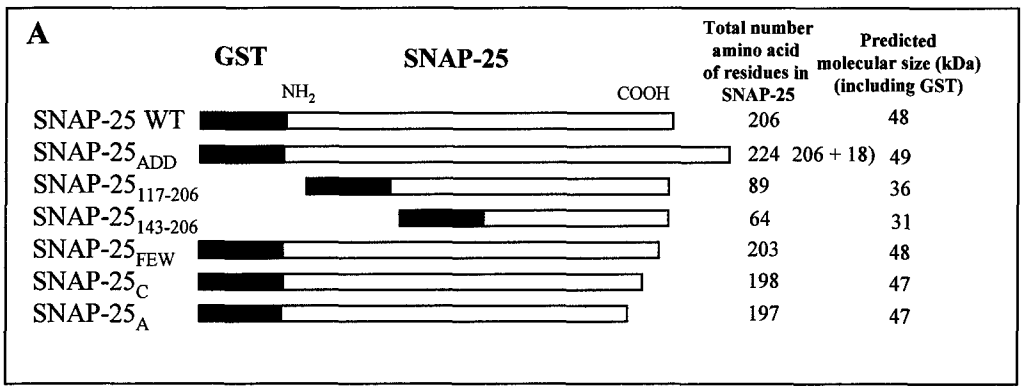
SNAP-25 NQTA
SNAP-25 NARA
SNAP-25 NAEA
SNAP-25 NQRL
SNAP-25 NKHA
SNAP-25FEW
SNAP-25A
SNAP-25C
SNAP-25 NAAA
SNAP-25 NAKA
SNAP-25 NWWA
SNAP-25 NAKL
SNAP-25 QAKA
Mr (kDa)
A
81.0
51.2
33.6
24.0
Coomassie stain
B
~50
anti-SNAP-25190-197-Ig
C
~ 50
anti-SNAP-25-Ig (C-terminus)


A
GST
SNAP-25
NH2
COOH
Total number amino acid of residues in SNAP-25
Predicted molecular size (kDa) (including GST)
SNAP-25 WT
SNAP-25ADD
SNAP-25117-206
SNAP-25143-206
SNAP-25FEW
SNAP-25C
SNAP-25A
206
224 206 + 18)
89
64
203
198
197
48
49
36
31
48
47
47

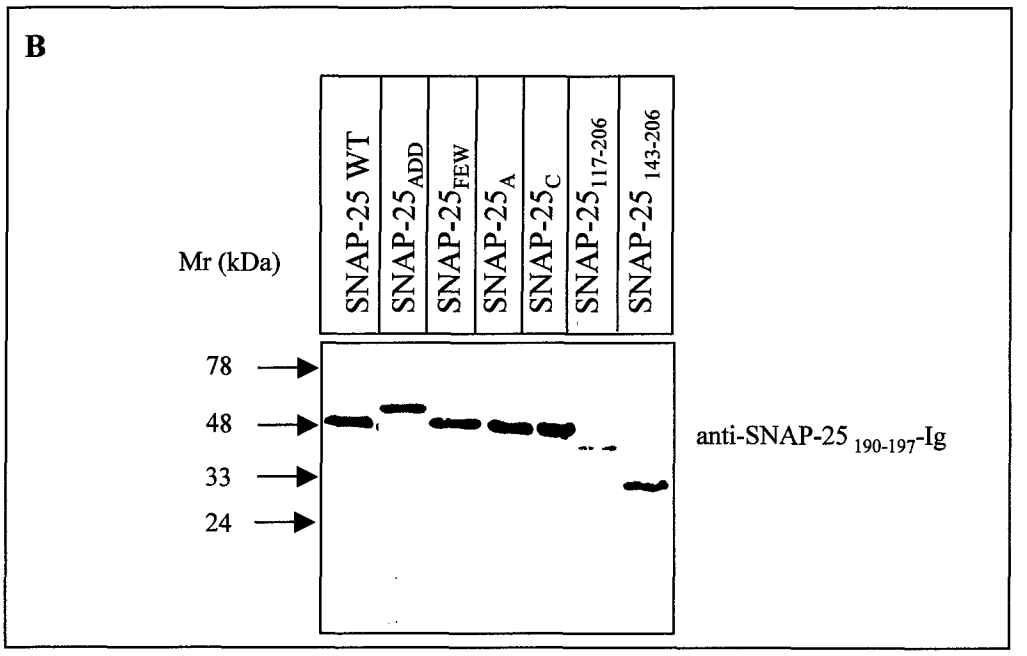

B
Mr (kDa)
SNAP-25 WT
SNAP-25ADD
SNAP-25FEW
SNAP-25A
SNAP-25C
SNAP-25117-206
SNAP-25 143-206
78
48
33
24
anti-SNAP-25 190-197-Ig

Figure 11

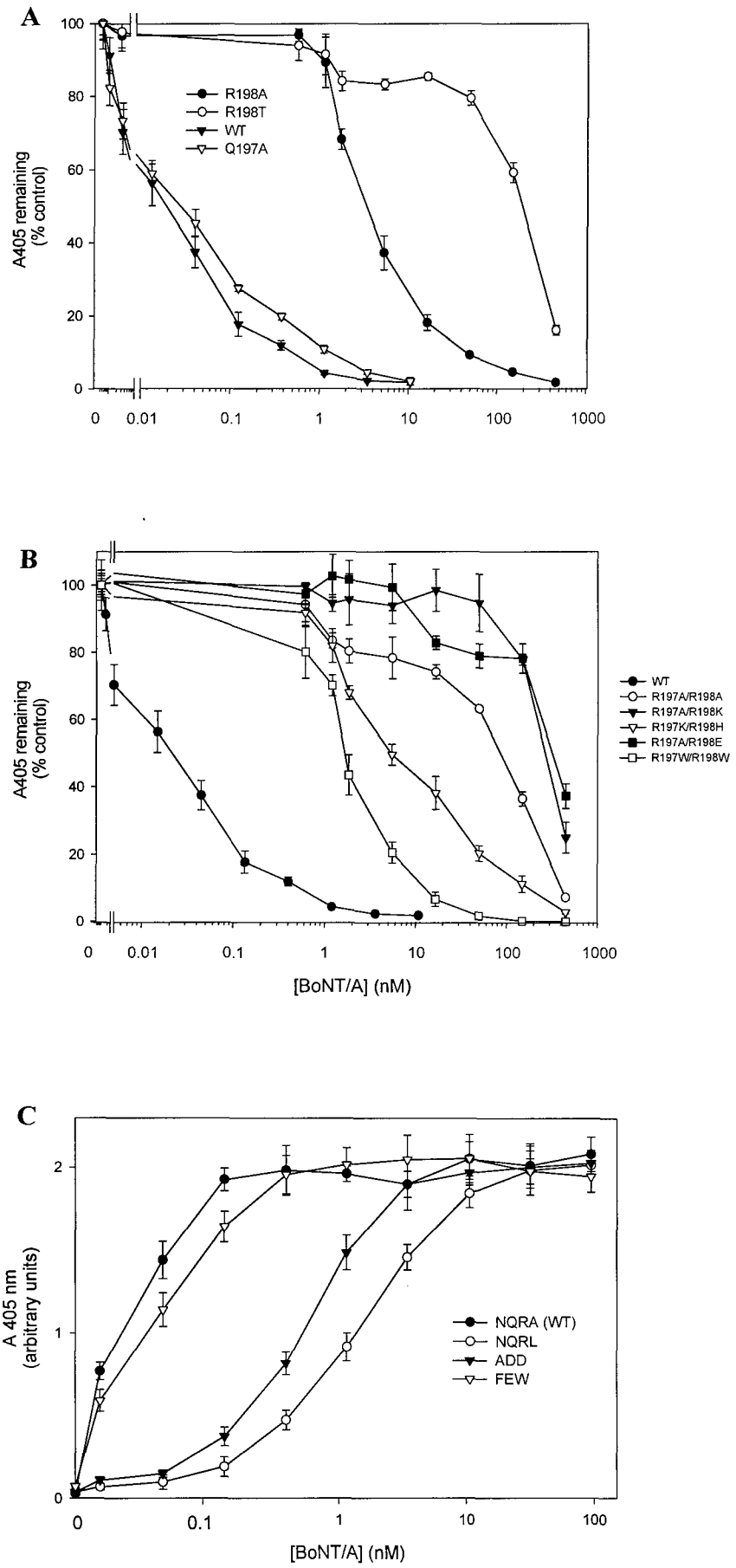

A
A405 remaining (% control)
R198A
R198T
WT
Q197A
B
A405 remaining (% control)
WT
R197A/R198A
R197A/R198K
R197K/R198H
R197A/R198E
R197W/R198W
[BoNT/A] (nM)
C
A 405 nm (arbitrary units)
NQRA (WT)
NQRL
ADD
FEW
[BoNT/A] (nM)

Figure 12

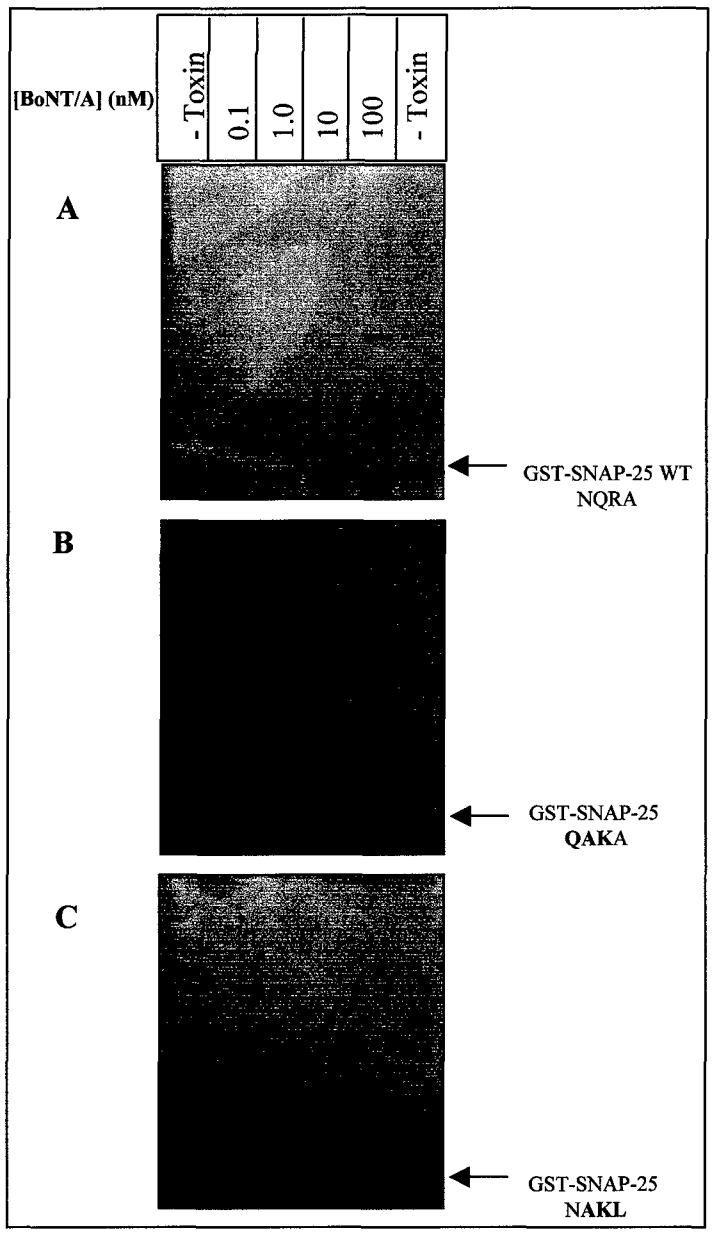
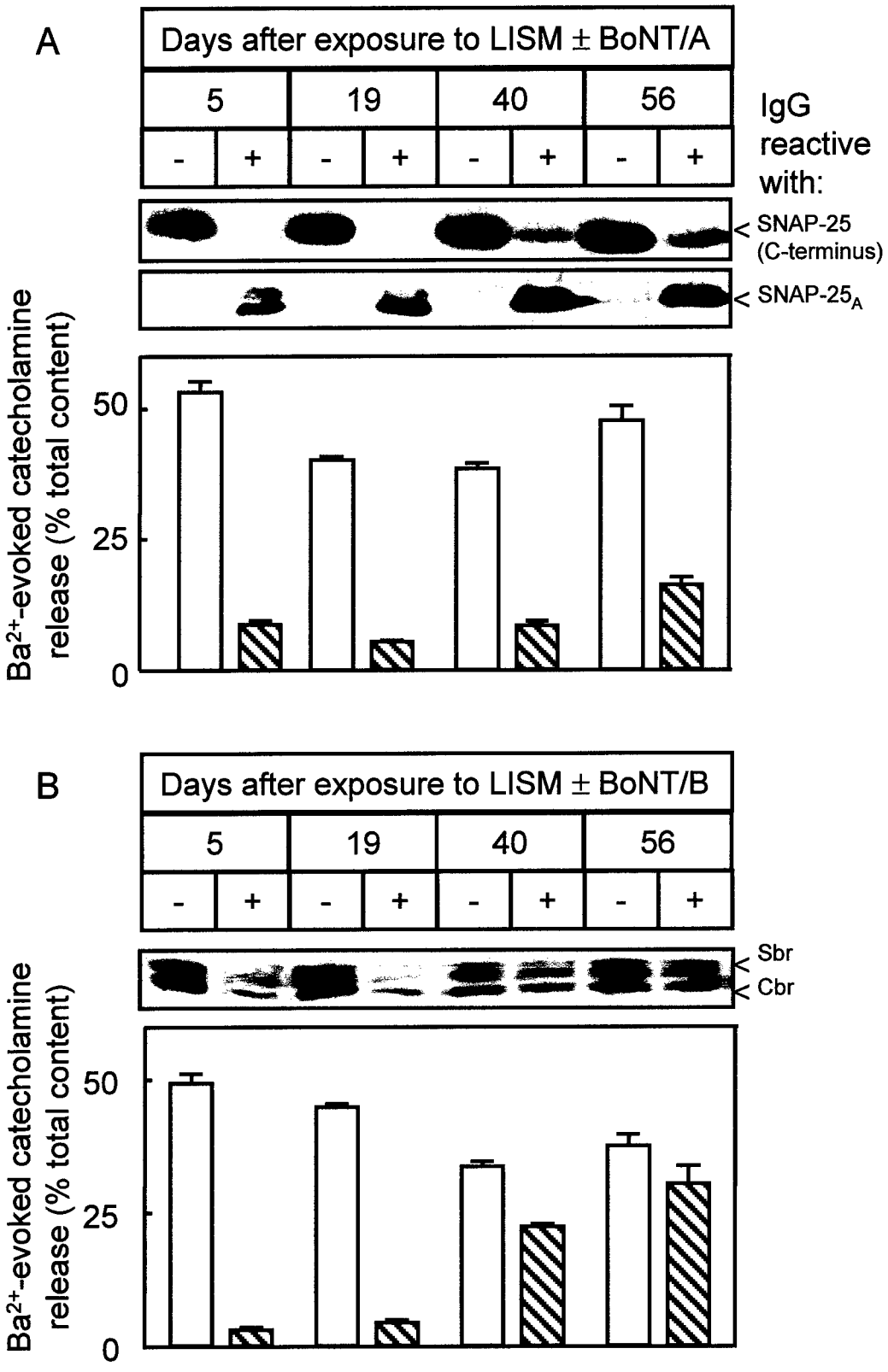

[BoNT/A] (nM)
- Toxin
0.1
1.0
10
100
- Toxin
A
GST-SNAP-25 WT
NQRA
B
GST-SNAP-25
QAKA
C
GST-SNAP-25
NAKL

Figure 13


A
Days after exposure to LISM ± BoNT/A
5
19
40
56
- + - + - + - +
IgG reactive with:
SNAP-25 (C-terminus)
SNAP-25A
Ba2+-evoked catecholamine release (% total content)
50
25
0
B
Days after exposure to LISM ± BoNT/B
5
19
40
56
- + - + - + - +
Sbr
Cbr
Ba2+-evoked catecholamine release (% total content)
50
25
0

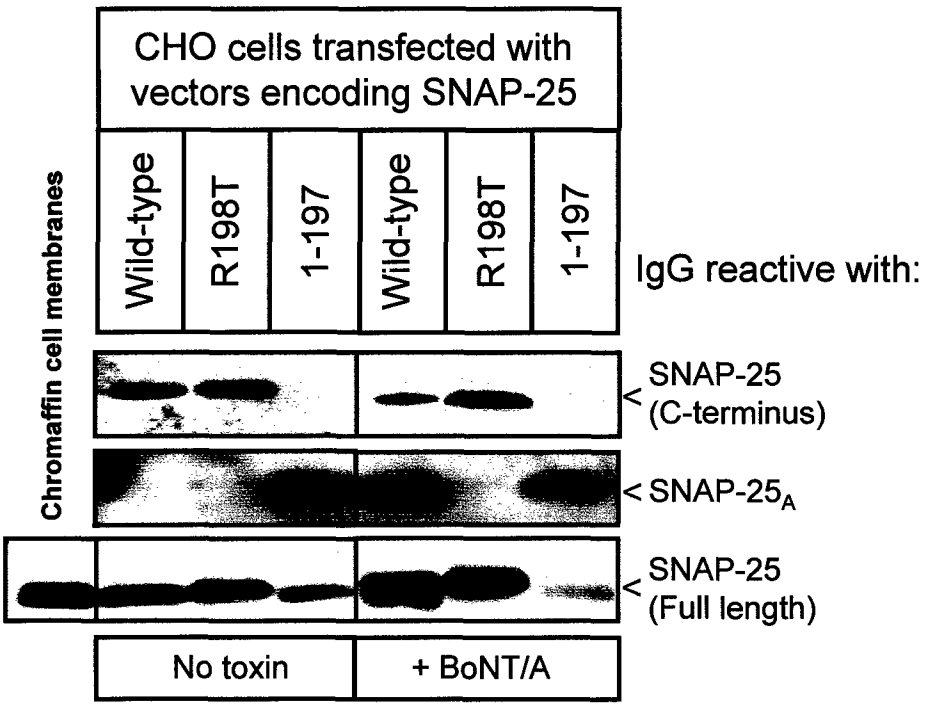

CHO cells transfected with vectors encoding SNAP-25
Wild-type
R198T
1-197
Wild-type
R198T
1-197
IgG reactive with:
Chromaffin cell membranes
SNAP-25 (C-terminus)
SNAP-25A
SNAP-25 (Full length)
No toxin
+ BoNT/A

Figure 15

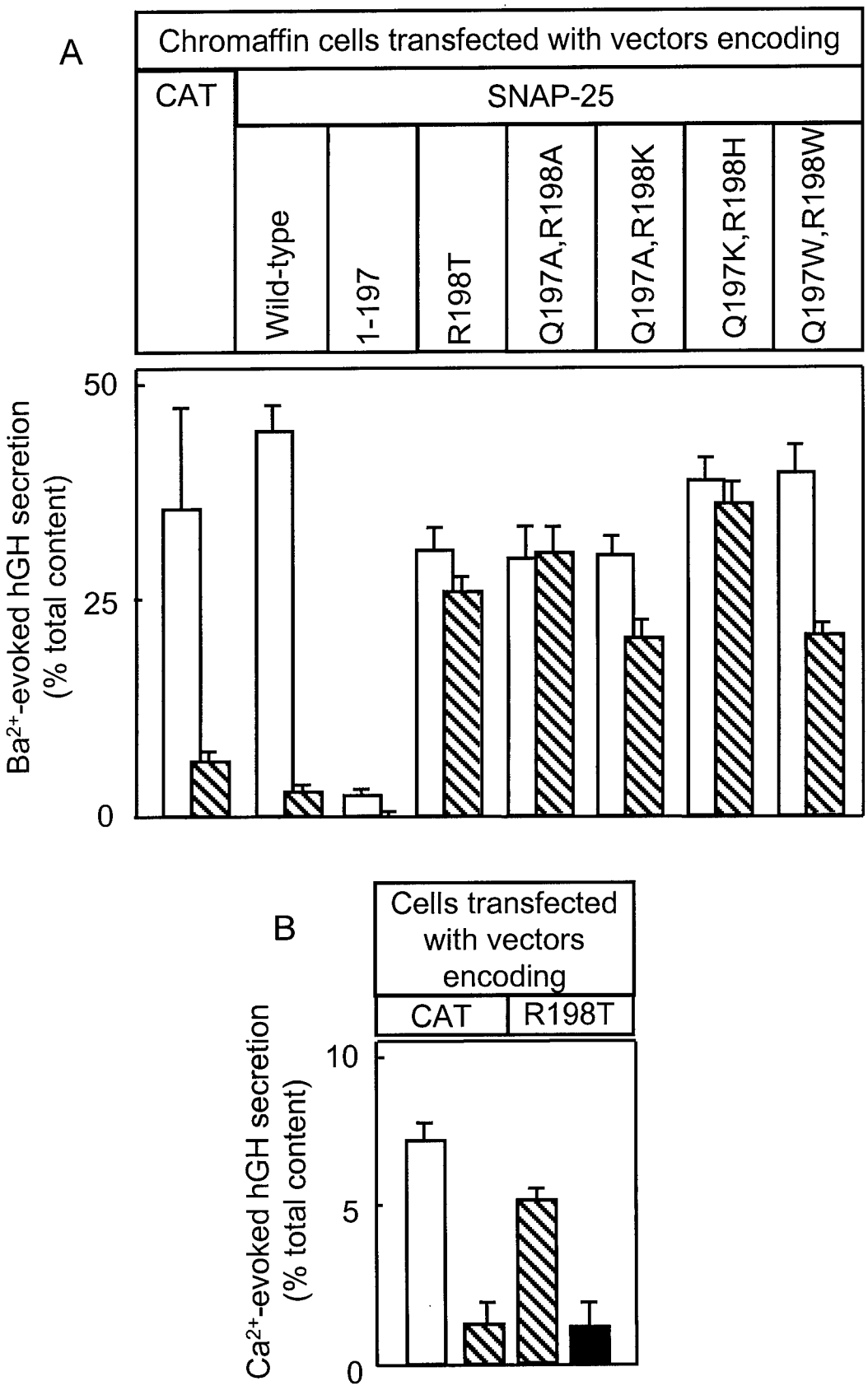

A
Chromaffin cells transfected with vectors encoding
CAT
SNAP-25
Wild-type
1-197
R198T
Q197A,R198A
Q197A,R198K
Q197K,R198H
Q197W,R198W
50
25
0
Ba2+-evoked hGH secretion
(% total content)
B
Cells transfected with vectors encoding
CAT
R198T
10
5
0
Ca2+-evoked hGH secretion
(% total content)

Figure 16

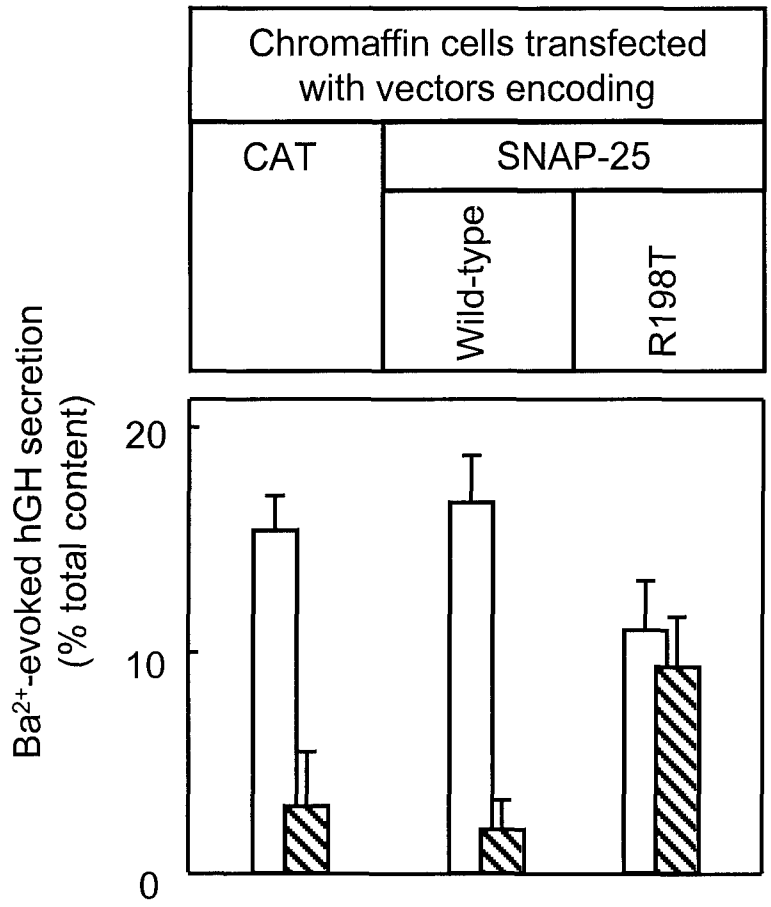
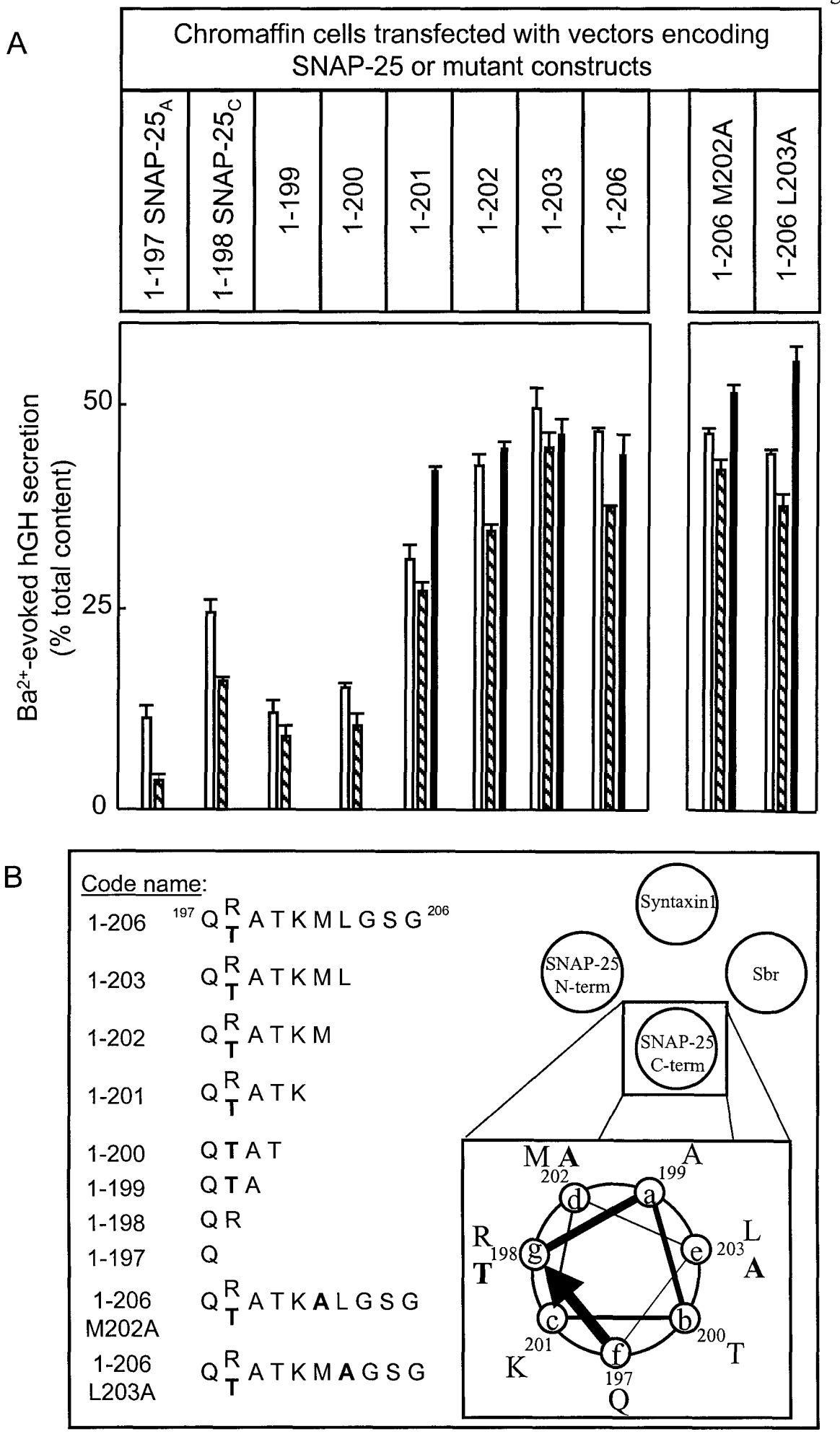
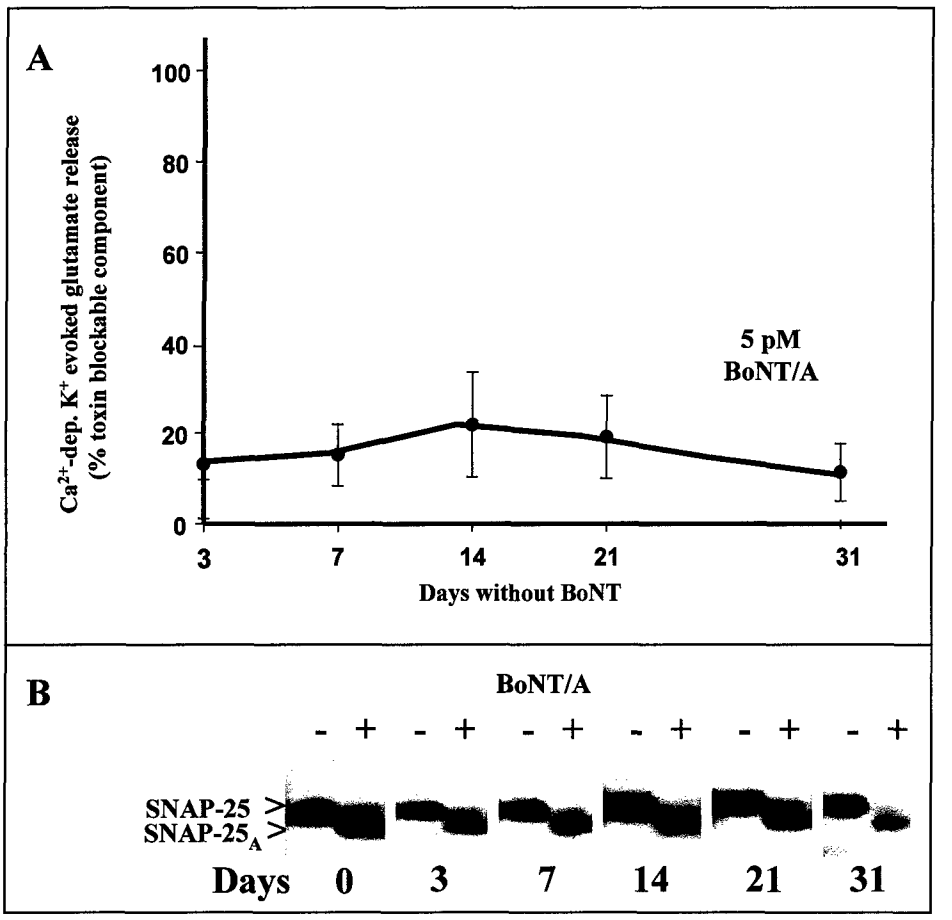

Chromaffin cells transfected with vectors encoding
CAT
SNAP-25
Wild-type
R198T
Ba2+-evoked hGH secretion (% total content)
20
10
0

Figure 17


A
Chromaffin cells transfected with vectors encoding SNAP-25 or mutant constructs
1-197 SNAP-25A
1-198 SNAP-25C
1-199
1-200
1-201
1-202
1-203
1-206
1-206 M202A
1-206 L203A
Ba2+-evoked hGH secretion (% total content)
50
25
0
B
Code name:
1-206 197 Q R/T A T K M L G S G 206
1-203 Q R/T A T K M L
1-202 Q R/T A T K M
1-201 Q R/T A T K
1-200 Q T A T
1-199 Q T A
1-198 Q R
1-197 Q
1-206 M202A Q R/T A T K A L G S G
1-206 L203A Q R/T A T K M A G S G
Syntaxin1
SNAP-25 N-term
Sbr
SNAP-25 C-term
M A 202
A 199
R T 198
L 203 A
T 200
K 201
Q 197
a
b
c
d
e
f
g


A
Ca2+-dep. K+ evoked glutamate release
(% toxin blockable component)
100
80
60
40
20
0
5 pM
BoNT/A
3
7
14
21
31
Days without BoNT
B
BoNT/A
- + - + - + - + - + - +
SNAP-25
SNAP-25A
Days 0 3 7 14 21 31

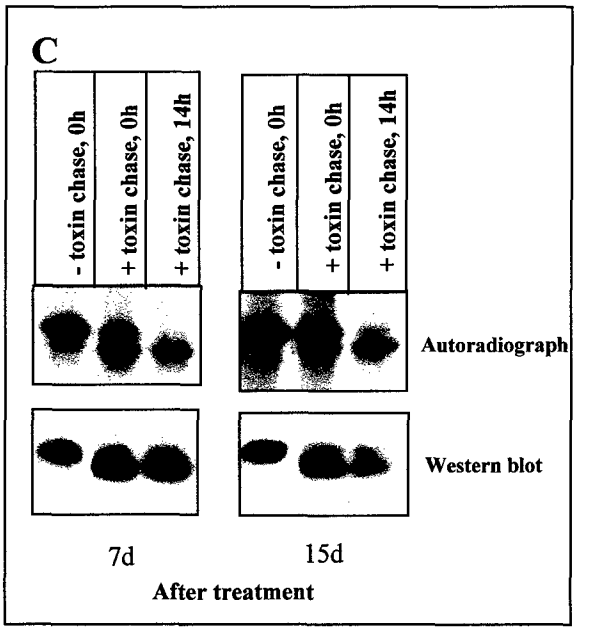

C
- toxin chase, 0h
+ toxin chase, 0h
+ toxin chase, 14h
- toxin chase, 0h
+ toxin chase, 0h
+ toxin chase, 14h
Autoradiograph
Western blot
7d
15d
After treatment

Figure 19

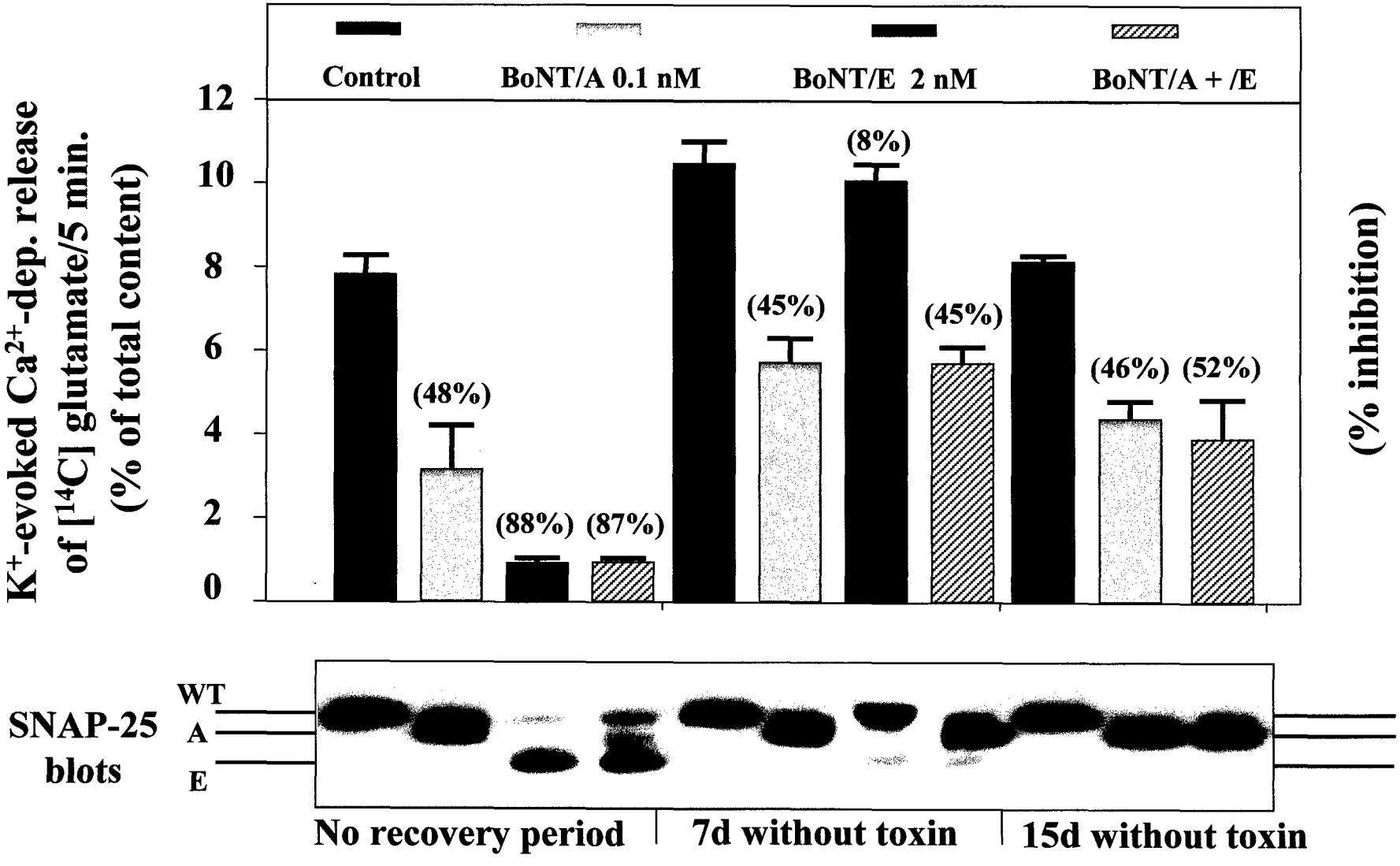
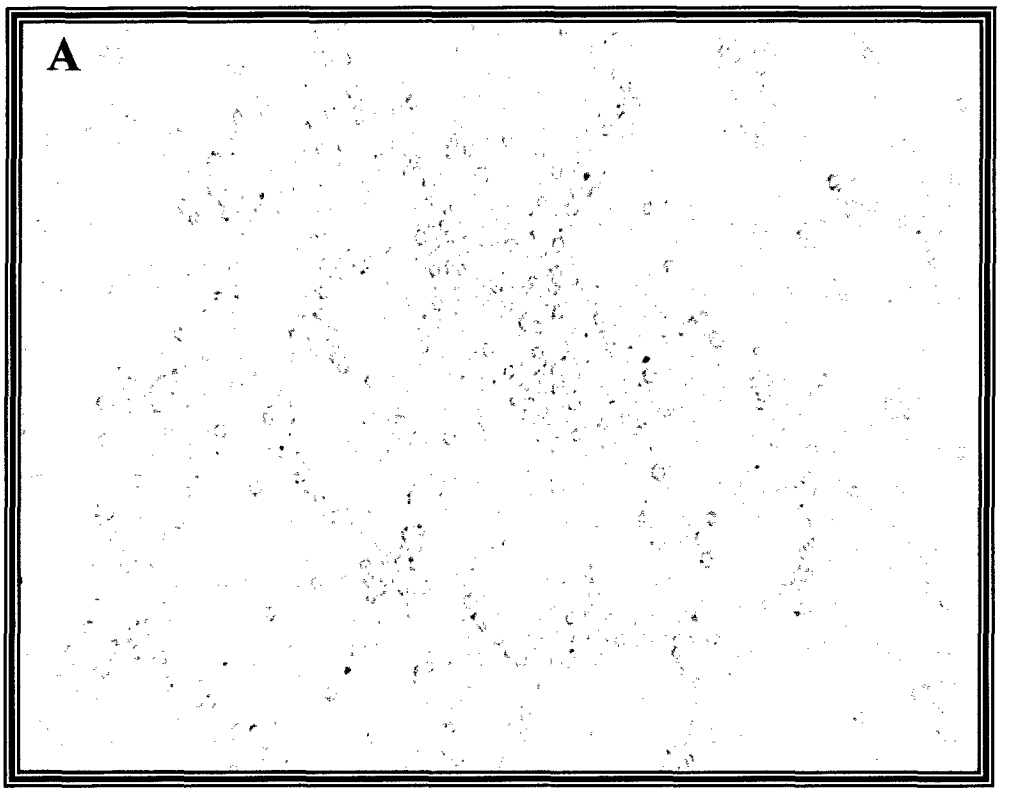

Control
BoNT/A 0.1 nM
BoNT/E 2 nM
BoNT/A + /E
K+-evoked Ca2+-dep. release of [14C] glutamate/5 min. (% of total content)
12
10
8
6
4
2
0
(48%)
(88%)
(87%)
(45%)
(8%)
(45%)
(46%)
(52%)
(% inhibition)
SNAP-25 blots
WT
A
E
No recovery period
7d without toxin
15d without toxin

Figure 20



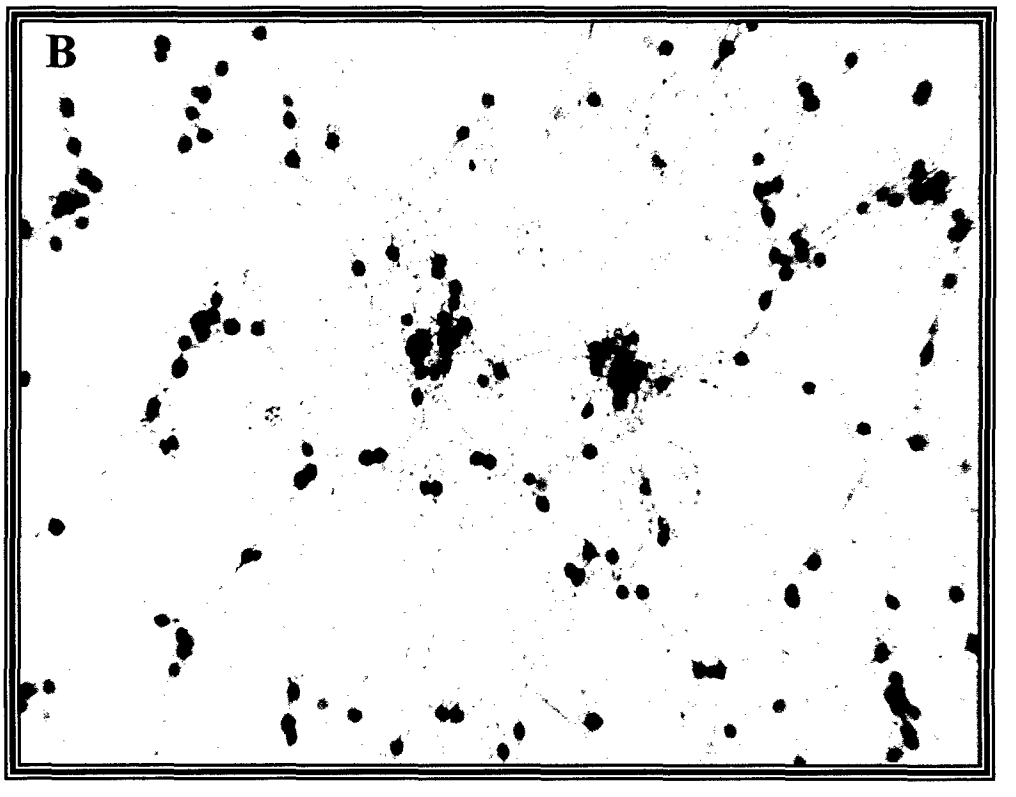
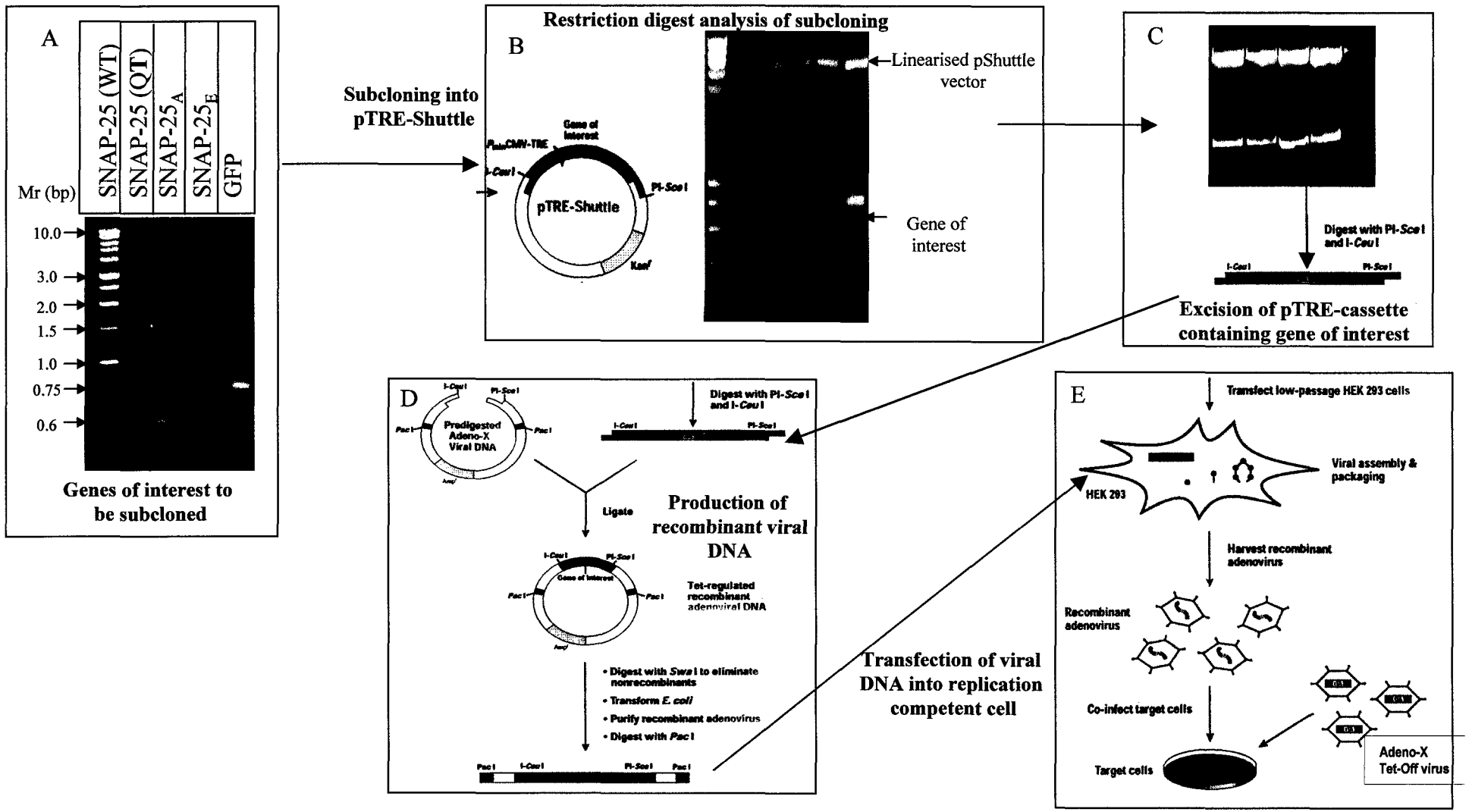
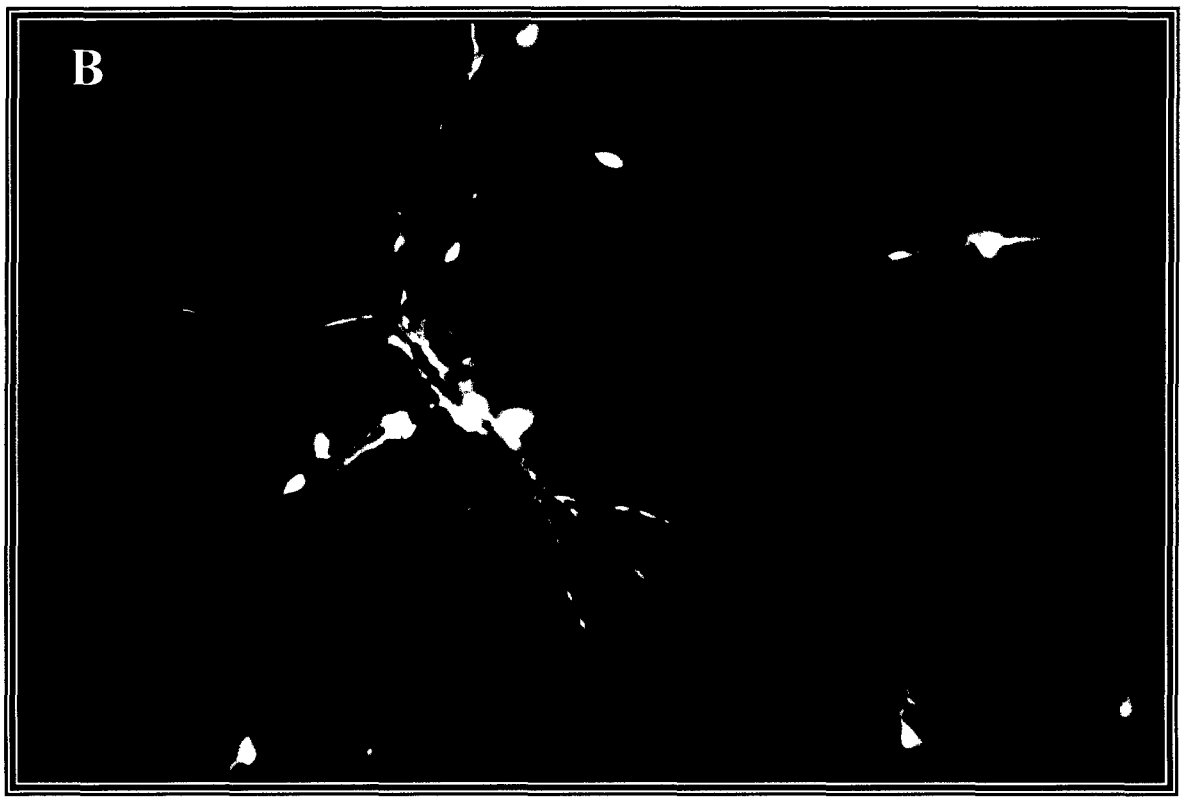


Figure 21


A
SNAP-25 (WT)
SNAP-25 (QT)
SNAP-25A
SNAP-25E
GFP
Mr (bp)
10.0
3.0
2.0
1.5
1.0
0.75
0.6
Genes of interest to be subcloned
Subcloning into pTRE-Shuttle
Restriction digest analysis of subcloning
B
Linearised pShuttle vector
Gene of interest
pTRE-Shuttle
C
Digest with PI-Sce I and I-Ceu I
Excision of pTRE-cassette containing gene of interest
D
Predigested Adeno-X Viral DNA
Digest with PI-Sce I and I-Ceu I
Ligate
Production of recombinant viral DNA
Tet-regulated recombinant adenoviral DNA
• Digest with Swa I to eliminate nonrecombinants
• Transform E. coli
• Purify recombinant adenovirus
• Digest with Pac I
Transfection of viral DNA into replication competent cell
E
Transfect low-passage HEK 293 cells
HEK 293
Viral assembly & packaging
Harvest recombinant adenovirus
Recombinant adenovirus
Co-infect target cells
Target cells
Adeno-X Tet-Off virus


B