# Rational modification of botulinum neurotoxin by directed scanning mutagenesis

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## Introduction

Botulinum neurotoxins (BoNTs) have found wide application as therapeutic agents for the treatment of a range of neuromuscular disorders due to their potency and specificity for neuronal cells[1]. They comprise three structurally and functionally distinct domains (LC, $H_N$, and $H_C$ domains) which may be modified to enhance/alter their activity; however, the challenge is to identify residues and regions in the molecule suitable for modification without disrupting BoNT function. To achieve this we have used selective lysine scanning mutagenesis (due to its suitability to chemical and biological coupling) and characterised the resulting molecules in *in vitro*, *ex vivo*, and *in vivo* assays to identify suitable positions for future modification.

### *Aims of the study*

- Rationally identify residues in BoNT/A heavy chain and BoNT/E light chain that are amenable to modification
- Review structural and literature knowledge to drive residue selection
- Assess single and multiple residue lysine substitutions
- Test predictions in functional assays to identify modifications which do not affect BoNT function

### *Benefits*

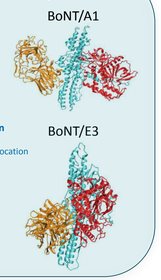
- To be able to modify BoNT molecules without interfering with core functional domains
- Allow for chemical coupling (small molecules) to BoNT
- Substitution of non-natural amino acids (stability)
- Identification of "hot spots" for other modifications

## Background

### *BoNT serotypes display a modular structure*

- Key functionality of light chain (LC, protease), N-terminus of heavy chain ($H_N$, translocation), and C-terminus of heavy chain ($H_C$, binding)
- Modularity of structure/function facilitates a rational approach to mutagenesis

**Figure 1.** BoNT/A1[5] and BoNT/E3[6] structure/function relationship




### *Selection of residues for lysine mutagenesis*

- Sequence analysis and review of literature data allowed for identification of regions to avoid
- Rational selection of residues to mutate was guided by a defined set of inclusion criteria

**Figure 2.** Exclusion and inclusion criteria for residue selection

Guidance from the literature about "No-Go" areas:
- Catalytic domain of the light chain
- Secondary structure
- Residues implicated in receptor binding (ganglioside, protein)

Inclusion criteria for residue modification
- Type of residue (polar preferred)
- Surface exposed
- Lysine modification does not introduce a ubiquitination site (CKSAAP_UbSite)
- Conservation across serotypes/subtypes (less conservation preferred)

## Methods

### *Structural/computational*

- Structural inspection and sequence alignments of BoNT/A and BoNT/E serotypes and subtypes were used in the selection of residues for modification

### *Molecular Cloning*

- *E. coli* codon optimised genes encoding BoNT/A1 or BoNT/E3 LC with an N-terminal Maltose Binding Protein (MBP) tag were used to generate the modified constructs
- Site directed mutagenesis was used to mutate selected residues to lysine

### *Protein Production*

- All proteins were expressed and purified from BL21(DE3) *E. coli*
- Molecules were purified by classical chromatography, exogenously activated, and finally exchanged into PBS buffer

### *Functional Assays of BoNT activity*

- LC potency was measured using the BoTest to assess SNAP-25 cleavage by BoNT/E3 light chain (BioSentinel)
- *In vitro* cellular potency was measured in primary embryonic spinal cord neurons (eSCN) prepared as described previously[2,3]. SNAP-25 cleavage potency was assessed by treatment of cells with BoNT/A1 for 24 hours and quantitation of full length and cleaved forms of SNAP-25 by Western Blot (Sigma #S9684)
- *Ex vivo* neuromuscular paralysis was measured by treating preparations of mouse phrenic nerve hemi-diaphragm with 10 pM BoNT/A1 and measuring time to 50% paralysis upon stimulation
- *In vivo* potency was assessed using an adapted mouse Digit Abduction Score (DAS) assay[4]. Potency (ED50) was estimated from regression analysis of the dose response

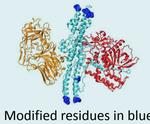
## Results

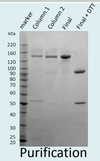
### *Lysine mutagenesis in the $H_N$ domain of BoNT/A1*

- Two combinations of 6 and 5 residues ($H_N$/A_1 and $H_N$/A_2, respectively) were substituted with lysine in the $H_N$ domain of BoNT/A1
- Molecules were assessed *in vitro* (eSCN) and *ex vivo* (hemi-diaphragm)
- $H_N$/A_1 was at least equipotent to unmodified BoNT/A1
- $H_N$/A_2 was significantly less potent than BoNT/A1 in both *in vitro* and *ex vivo* assays

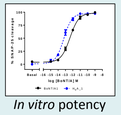
**Figure 3.** Characterisation of BoNT/A1 molecules with lysine substitutions in the $H_N$ domain

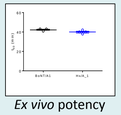
| Molecule | Modifications |
|---|---|
| $H_N$/A_1 | N476K, N763K, N687K, E599K, I831K, N761K |


Modified residues in blue

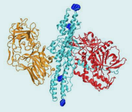


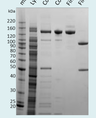
Purification


*In vitro* potency

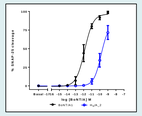

*Ex vivo* potency

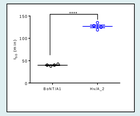
| Molecule | Modifications |
|---|---|
| $H_N$/A_2 | N578K, V675K, I685K, T755K, E757K |


Modified residues in blue



Purification


*In vitro* potency

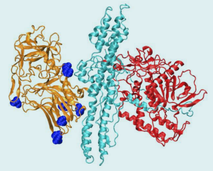

*Ex vivo* potency

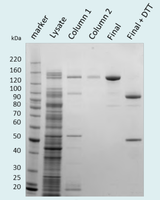
### *Lysine mutagenesis in the $H_C$ domain of BoNT/A1*

- Seven residues were substituted with lysine in the $H_C$ domain of BoNT/A1 ($H_C$/A_1)
- $H_C$/A_1 was assessed *in vitro* (eSCN), *ex vivo* (hemi-diaphragm), and *in* vivo (mouse DAS)
- In all assays, $H_C$/A_1 was equipotent to BoNT/A1

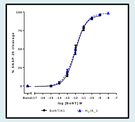
**Figure 4.** Characterisation of BoNT/A1 with 7 lysine substitutions in the $H_C$ domain

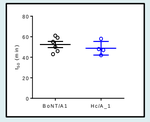
| Molecule | Modifications |
|---|---|
| $H_C$/A_1 | N886K, N930K, S955K, Q991K, N1026K, N1052K, Q1229K |


Modified residues in blue



Purification


*In vitro* potency


*Ex vivo* potency

| Molecule | DAS $ED_{50}$ (pg/mouse) |
|---|---|
| BoNT/A1 | 2.0 |
| $H_C$/A_1 | 1.2 |

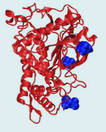
*In vivo* potency

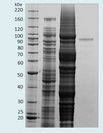
### *Lysine mutagenesis in the LC of BoNT/E3*

- Three residues were substituted in the BoNT/E3 light chain (LC/E3_1)
- To aid in solubility, LC/E3_1 contained an MBP tag
- LC/E3_1 was assessed via the BoTest and found to be equipotent to unmodified LC/E3

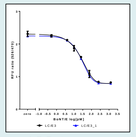
**Figure 5.** Characterisation of BoNT/E3 LC with 3 lysine substitutions in the LC domain

| Molecule | Modifications |
|---|---|
| LC/E3_1 | Q123K, N138K, Q237K |


Modified residues in blue



Purification


LC Potency

## Conclusions

In the absence of a complete understanding of the structure-function relationship of BoNT domains, we used rational scanning mutagenesis in regions of interest to identify potential sites for modification. We demonstrated the feasibility of the approach by assessing their impact in a suite of assays to evaluate the modified molecules.

- Residues were identified in all domains which, when substituted in combination, had no effect on potency
- Groups of residues were also identified in $H_N$/A which significantly impaired potency

The ability to identify suitable sites for mutation will enable future modification of BoNT molecules with greater confidence.

### *References*

1. Chen *et al* (2013) Pharmacotherapy 33(3):304
2. Masuyer *et al* (2011) J Struct Biol 174:52
3. Fonfria *et al* (2016) J Recept Signal Transduct Res 36(1):79
4. Aoki *et al* (1999) Eur J Neurol 6:53
5. Lacy *et al* (1998) Nat Struct Biol 5(10):898
6. Kumaran *et al* (2009) J Mol Biol 386:233

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