rBoNT/A mutants with polypeptide extension show different kinetic properties

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Botulinum Toxin Research
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Introduction

- So far, seven serotypes of botulinum neurotoxins (BoNTs) are known, all of which vary with respect to onset and duration of paralytic effect.[1]
- In particular, BoNT/A and BoNT/E differ markedly in their kinetic profiles; the reasons for this remain elusive.[2]
- The fate of the enzymatically active light chain (LC) of BoNT inside the neuron might be responsible for the course of paralysis caused by the inhibition of neurotransmitter release, which in the case of BoNT/A and BoNT/E is due to cleavage of synaptosome associated protein 25 (SNAP-25).[3]
- Modulation of the pharmacologic properties of pharmaceutically relevant proteins could be effected via the addition of unstructured tails, such as polyethylene glycol (PEG) or hydroxylethyl starch (HES) moieties.[4]
- Polypeptide extensions with unstructured character may be able to mimic this effect while amino acid substitutions in the polypeptide extension may allow for specific fine-tuning.

Aim

- To generate recombinant BoNT/A1 variants (rBoNT/A) with polypeptide extensions at the N-terminus of the LC and to characterize the pharmacologic properties, such as onset and duration of effect.

Methods

Preparation of BoNT/A variants

- Codon-optimized gene constructs were cloned into pET29c for expression in *Escherichia coli*, harboring N- and C-terminal His6- and Strep-affinity tags, respectively. Expression of modified rBoNT/A variants was performed in Riesenberg minimal medium.
- Soluble proteins containing supernatants of crude extracts were obtained after cell disruption (French Press) and centrifugation (20,000 rpm, 30 minutes, 4 °C).
- Protein purification was carried out using a three-step purification protocol:
  - immobilized metal affinity chromatography
  - Strep-affinity chromatography
  - size-exclusion chromatography
- Preparations of rBoNT/A were activated with thrombin yielding >99% of di-chain toxin. Tags were cleaved off during activation.
- Toxin concentrations were determined by sandwich enzyme-linked immunosorbent assay (ELISA) measurements.

Voluntary mouse running assay (MRA)

- MRA was carried out using REVOLYZER® (preclinics GmbH, Germany) systems.
- Running distance was evaluated as a half-hourly average and recorded in daily intervals. One group was used as positive control and injected with incobotulinumtoxinA (Xeomin®, Merz Pharmaceuticals GmbH, Germany) at 0.6 U (3 pg).
- Test substance and positive control were applied to achieve equal paralysis of 80–90% (i.e. 10–20% residual running activity).
- Test substance was administrated via intramuscular injections of 20 µL into the gastrocnemius muscle of the right hind limb.[2] The groups comprised eight animals each.

Results

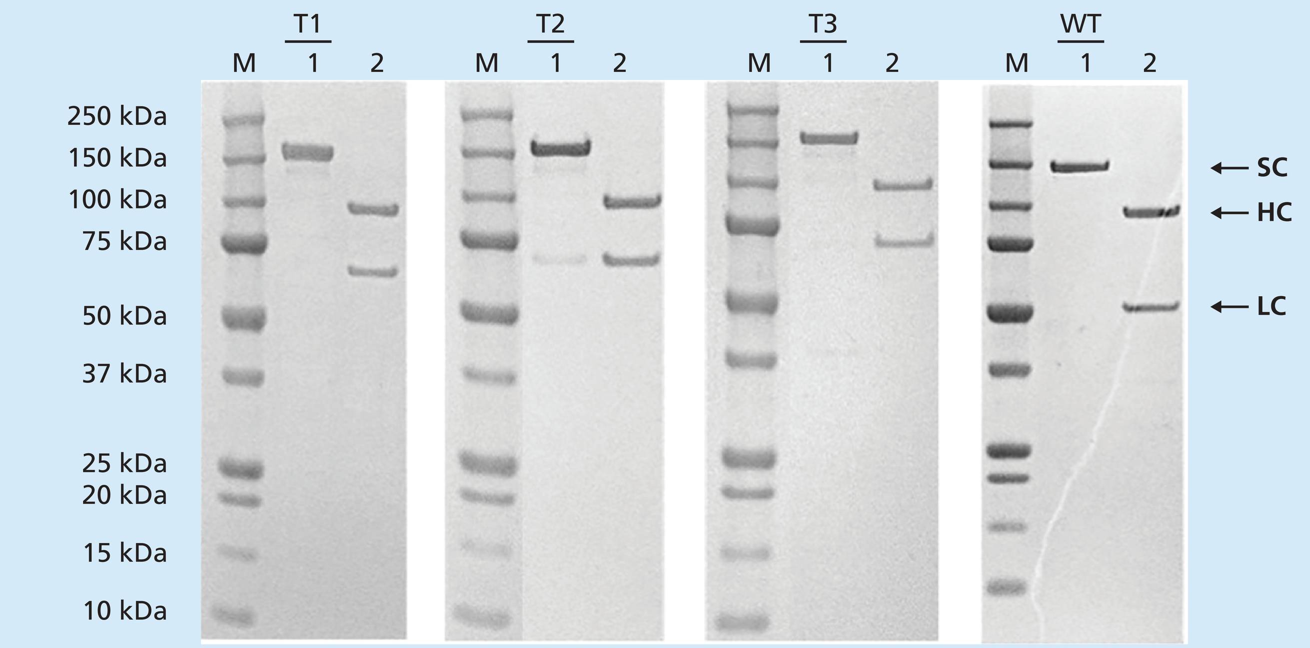
- A total of three rBoNT/A variants – T1, T2, and T3 – with polypeptide extensions of 100 amino acids in length at the N-terminus of the LC (**Table 1**) were successfully produced in *E. coli* and purified.

Table 1. Overview of recombinant BoNT/A variants

Acronym	Polypeptide extension
T1	(VTPVVPVPVT PVVPVPTVPV) ×5
T2	(GTPGGPGPGT PGGPGPTGPG) ×5
T3	(GSPGGPGPGS PGGPGPSGPG) ×5

- Proteolytic cleavage with thrombin yielded highly pure rBoNT/As in di-chain form without tags (**Figure 1**).

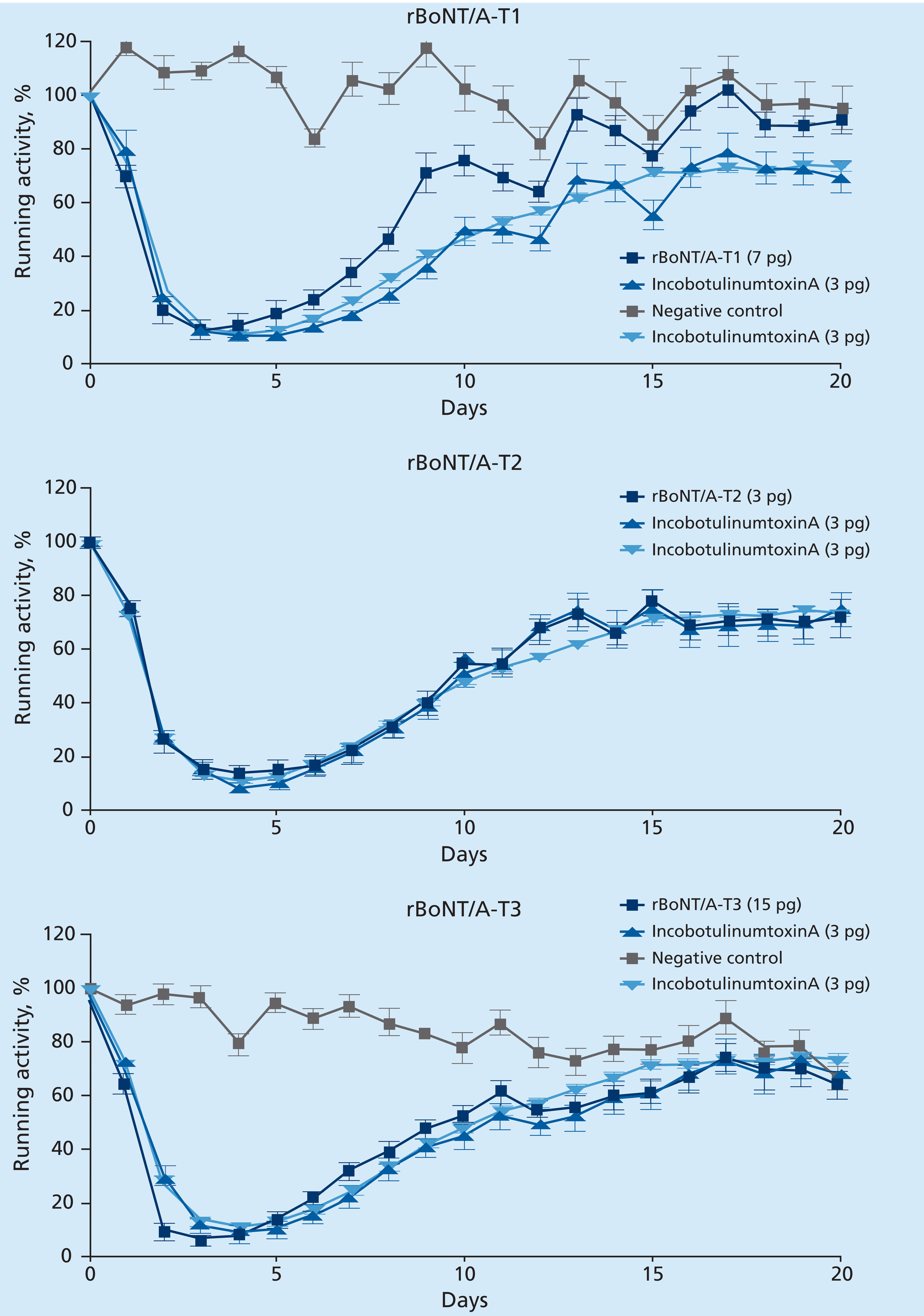
Figure 1. Activation of recombinant BoNT/A variants used in this study


T1
T2
T3
WT
M 1 2
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
10 kDa
← SC
← HC
← LC

Recombinant modified BoNT/A constructs were expressed in *E. coli* as SC proteins. Cleavage by thrombin between LC and HC results in activation of the protein.
Results of purification (1) and after activation (2) are shown. The disulfide bond between LC and HC was reduced using β-mercaptoethanol.
BoNT, botulinum neurotoxin; HC, heavy chain; LC, light chain; M, molecular weight marker; SC, single chain.

- Voluntary MRAs were utilized to assess the time course of paralytic activity *in vivo* (**Figure 2**).
- A shorter duration of paralysis compared to incobotulinumtoxinA was observed for rBoNT/A-T1. No difference to incobotulinumtoxinA was observed for rBoNT/A-T2 (Val to Gly substitution), while additional substitution of Thr to Ser (rBoNT/A-T3) resulted in an earlier onset of the paralysis effect.

Figure 2. Results of voluntary MRAs


rBoNT/A-T1
Running activity, %
Days
0
5
10
15
20
120
100
80
60
40
20
rBoNT/A-T1 (7 pg)
IncobotulinumtoxinA (3 pg)
Negative control
IncobotulinumtoxinA (3 pg)
rBoNT/A-T2
rBoNT/A-T2 (3 pg)
IncobotulinumtoxinA (3 pg)
IncobotulinumtoxinA (3 pg)
rBoNT/A-T3
rBoNT/A-T3 (15 pg)
IncobotulinumtoxinA (3 pg)
Negative control
IncobotulinumtoxinA (3 pg)

The paralysis effects of recombinant BoNT/A variants (blue square) were analyzed in this assay. IncobotulinumtoxinA (BoNT/A WT; blue triangle) was used as an internal positive control. The negative control group (if available) comprised mice that were not injected with any substance (grey square). IncobotulinumtoxinA (average of 16 assays; light blue inverted triangle) consists of mean and standard deviation of 16 individual assays. T1 shows a slightly shortened duration of the paralysis effect with faster recovery. T3 shows a somewhat earlier onset of paralysis effect. The small sequence deviation between T2 and T3, i.e. Thr versus Ser, modulates comparability to the WT.
BoNT, botulinum neurotoxin; MRA, mouse running assay; WT, wild type.

Conclusions

- Genetic engineering and recombinant production of rBoNT/A1 variants proved to be a versatile tool for extending the amino acid sequence at the N-terminus of the LC.
- Based on this approach, we were able to generate N-terminally modified LC variants that show an earlier onset of the paralysis effect (rBoNT/A-T3) or a faster recovery (rBoNT/A-T1).
- Remarkably, the amino acid sequence of rBoNT/A-T2, despite being close to rBoNT/A-T3 and rBoNT/A-T1, did not result in significantly altered pharmacologic properties of the molecule.
- This shows that sequence modifications are not necessarily advantageous unless the molecular background is understood.
- The cause of the observed effect is not yet known, but paves the way for further investigation and optimization. Similarly, it highlights the potential of polypeptide extension in the modification of biopharmaceuticals.[5]

References

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Acknowledgments

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