Use of high-content imaging in botulinum toxin research

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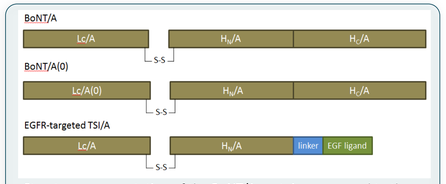
Introduction

- SNAP-25 cleavage is the last step in the mechanism of action of BoNT/A and can be quantified to characterise the activity of BoNT/A and BoNT/A-derived Targeted Secretion Inhibitors (TSI), in which the neuronal targeting domain of BoNT/A has been substituted with a cell targeting domain for the cell type of choice.
- High content imaging (HCI) has emerged as an alternative method that may provide mechanistic insights into the action of BoNTs and TSIs.
- The objective of this work was to explore HCI as an assay of protein localisation and relate the HCI results to those from SNAP-25 cleavage.



Structure representation of the BoNT/A proteins compared to the TSI/A protein. The enzymatic light chain (Lc/A), the translocation domain (HN/A) and the binding domains (EGF or HC/A) are shown.

References

Fernandez-Salas *et al.* (2012) PLOS ONE 7 (11), e4951
Fonfria *et al.* (2016). J Recept Signal Transduct Res, 36 (1): 79-88
Masuyer *et al.* (2009) BBRC 381, 50-3;
Masuyer *et al.*(2011) J. Struct. Biol 174, 52-57
Somm *et al.* (2012) J.Clin.Investig. 9, 3295-306

Methods

BoNT/A and TSI/A proteins

- BoNT/A was purchased from Metabiologics Inc (Madison, WI, USA) or List Biological Laboratories Inc (Campbell, CA, USA).
- Epidermal growth factor receptor (EGFR)-targeted TSI/A and catalytically inactive BoNT/A (termed BoNT/A(0)) were manufactured at Ipsen Bioinnovation with modifications of previously described methods (Masuyer et al, 2009; Masuyer et al 2011, Somm et al, 2012).

Cell culture and treatment

- Human neuroblastoma SiMa cells (ACC, DSMZ, Braunschweig, Germany), used previously to study BoNT biology (Fernandez-salas et al, 2012) were cultured in RPMI-1640 media + 10% FBS at 37 °C in a humidified environment containing 5% CO_2. Human fibrosarcoma HT-1080 cells (ATCC, Teddington, UK) were cultured in DMEM/F12 media + 10 % FBS and 15 mM HEPES. For further details see Fonfria et al, 2016.
- Cortical (CTX) cultures followed largely the method described by Masuyer et al (2011). All procedures ensured humane treatment of the laboratory animals, according to applicable legislation. Briefly, cells were harvested from 15-18 day old embryos. The isolated tissue was digested by papain and then mechanically dissociated. Cells were plated into 96-well plates and placed in an incubator at 37 °C in a humidified CO_2 atmosphere. Cells were allowed to differentiate in culture with bi-weekly changes of growth medium before they were used for experiments (at least 14 days in culture).
- Cells were exposed to 10-300 nM BoNT/A, BoNT/A(0) or EGFR-targeted TSI/A for 30 min to 48 h, as indicated. When specified, cells were pre-treated for 30 min with 250 nM concanamycin A (ConA; Sigma, Poole, UK).

SNAP-25 cleavage assay

- Following treatment, cells were harvested in NuPAGE lysis buffer (Life Technologies) supplemented with DTT and benzonase (Sigma), and tested for SNAP-25 cleavage by Western blot.
- SNAP-25 was detected using a polyclonal antibody that detects both the full length and BoNT/A-cleaved forms (Sigma #S9684). We used anti-rabbit HRP (Sigma #A6154) as the secondary antibody.

High content screening analysis

- Following treatment, cells were fixed with 4% formaldehyde, washed and permeabilised with 0.5 mg/ml digitonin (Sigma). Hoechst 33342 was used to stain the nuclei. Primary antibodies were used against BoNT/A Lc (custom made at Eurogentec for Ipsen Bioinnovation), early endosome antigen 1 (EAA1, Santa Cruz Biotechnology, TX, USA) or SV2 (Abcam). Alexa Fluor-488 goat anti-rabbit or Alexa Fluor-594 donkey anti-rabbit or anti-goat (Sigma) were used as secondary antibodies.
- Images were acquired and analysed using an ImageXpress Micro XLS (Molecular Devices, Wokingham, UK).

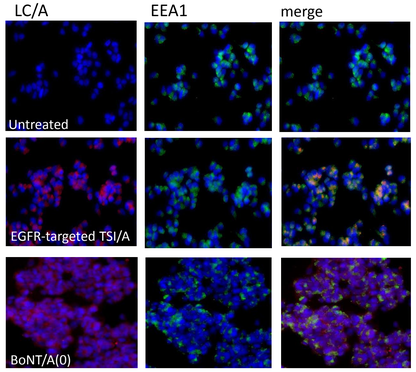
Conclusions

- Biochemical methods and HCI are complimentary techniques to unravel the complexity of BoNT & TSI biology.
- BoNT/A and the BoNT/A derived EGFR-targeted TSI/A have different endocytic routes. Entry of BoNT/A into neuroblastoma cell lines seems to follow the same endocytic pathway as in neurones, while early endosomes contribute to the entry of EGFR-targeted TSI/A in neuroblastoma and non-neuronal cells.
- HCI allows the study of endocytic routes in a high throughput and user-friendly manner.

Results

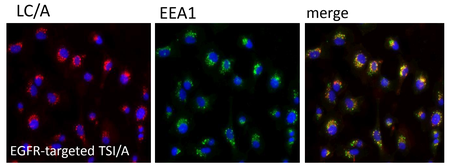
High content imaging reveals different trafficking routes for BoNT/A(0) and the BoNT/A-derived TSI

SiMa cells



In SiMa cells EGFR-targeted TSI/A internalized in a compartment which partially overlaps with early endosomes, whereas BoNT/A(0) does not. Blue: nuclear staining

HT1080 cells

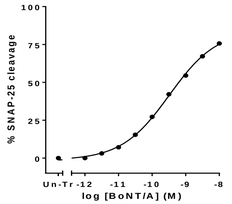


In non-neuronal HT-1080 cells the EGFR-targeted TSI/A also internalises in a compartment which partially overlaps with early endosomes. Blue: nuclear staining

In SiMa cells, BoNT/A and the BoNT/A derived TSI cleave SNAP-25 with different potencies. In cortical neurons, BoNT/A cleaves SNAP-25 with high potency

SiMa cells

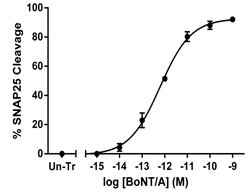
BoNT/A potency in SiMa cells after 24 h exposure
pEC_{50} was 9.48 ± 0.04 (n=3)



Note that 24 h treatment of SiMa cells with 1 µM EGFR-targeted TSI/A resulted in SNAP-25 cleavage of only 30.42 ± 8.32 % (n=3)

Rat primary cortical neurons

BoNT/A potency in cortical neurons after 24h exposure
pEC_{50} was 12.32 ± 0.10 (n=4)

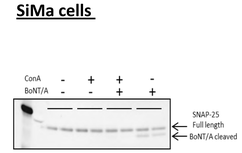


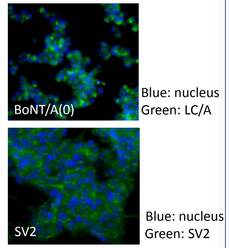
Un-Tr = untreated cells.

SNAP-25 and imaging studies reveal that BoNT/A enters into neuroblastoma cells and neurons via compartments that are acidifying and containing synaptic vesicle proteins

- **Interfering with acidification of compartments through con A interferes with SNAP-25 cleavage of BoNT/A**
- **BoNT/A(0) partitions to compartments that are labelled for Synaptic vesicle protein 2**

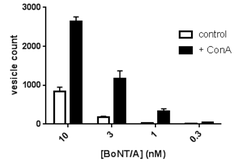
SiMa cells

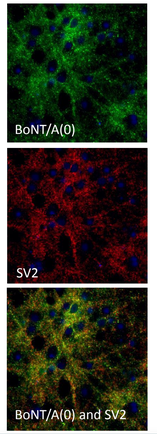




Blue: nucleus
Green: LC/A
Blue: nucleus
Green: SV2

Rat primary cortical neurons





Blue: nucleus
Green: LC/A,
Red: SV2

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