Research Article

Botulinum Neurotoxin Dose-Dependently Inhibits Release of Neurosecretory Vesicle-Targeted Luciferase from Neuronal Cells

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Summary

Botulinum toxin is a bacterial toxin that inhibits neurotransmitter release from neurons and thereby causes a flaccid paralysis. It is used as drug to treat a number of serious ailments and, more frequently, for aesthetic medical interventions. Botulinum toxin for pharmacological applications is isolated from bacterial cultures. Due to partial denaturation of the protein, the specific activity of these preparations shows large variations. Because of its extreme potential toxicity, pharmacological preparations must be carefully tested for their activity. For the current gold standard, the mouse lethality assay, several hundred thousand mice are killed per year. Alternative methods have been developed that suffer from one or more of the following deficits: *In vitro* enzyme assays test only the activity of the catalytic subunit of the toxin. Enzymatic and cell based immunological assays are specific for just one of the different serotypes. The current study takes a completely different approach that overcomes these limitations: Neuronal cell lines were stably transfected with plasmids coding for luciferases of different species, which were N-terminally tagged with leader sequences that redirect the luciferase into neuro-secretory vesicles. From these vesicles, luciferases were released upon depolarization of the cells. The depolarization-dependent release was efficiently inhibited by botulinum toxin in a concentration range (1 to 100 pM) that is used in pharmacological preparations. The new assay might thus be an alternative to the mouse lethality assay and the immunological assays already in use.

Keywords: Botulinum toxin, cell-based assay, mouse lethality assay

1 Introduction

Botulinum neurotoxin (BoNT) is a bacterial exotoxin that is produced by the strictly anaerobic spore-forming bacterium *Clostridium botulinum*. There are eight serotypes of Clostridium botulinum (Dover et al., 2014). The bacteria thrive in poorly sterilized canned food in which the heat-labile toxin accumulates and can cause severe food poisoning (Erbguth, 2008).

BoNT is a potent neurotoxin (Hakami et al., 2011). It inhibits the release of neurotransmitters from the presynaptic buds and thereby impairs the signal transmission between nerves or between nerves and skeletal muscle at the motor end plate. It thereby causes a flaccid paralysis resulting in death due to asphyxia. The toxin consists of two subunits. The large subunit binds to the cell surface of nerve cells and is responsible for tissue specificity (Aoki and Guyer, 2001). It permits the uptake of the small subunit of the toxin into the cell. The small subunit harbors a protease activity that cleaves proteins involved in the fusion of the neurosecretory neurotransmitter-containing vesicles with the presynaptic membrane. The different serotypes produce different proteases with differing substrate specificities (Aoki and Guyer, 2001).

When applied locally at very low doses, botulinum toxins of serotype A and B can be used as a therapeutic for a number of ailments including torticollis spasmodicus, blepharospasm, strabism, facial spasms or hyperhidrosis (Chen, 2012; Lakraj et al., 2013; Sesardic and Das, 2009). In recent years, however, the major area of application of botulinum toxin serotype A has been aesthetic medicine, in particular to remove facial wrinkles that result from the contraction of subcutaneous muscles (Chen, 2012).

BoNT for medical use is purified from bacterial cultures. During the purification procedure varying fractions of the large and small subunit, both of which are essential for biological activ-

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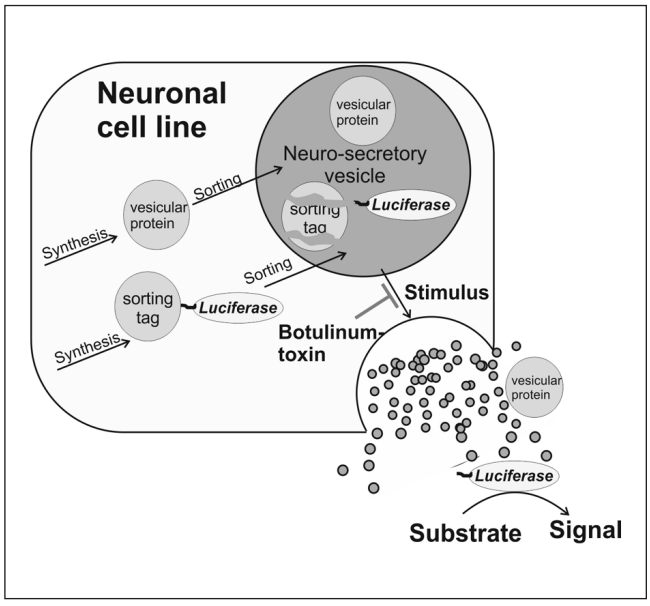

Fig. 1: Schematic view of the test principle
A luciferase is N-terminally tagged with a peptide sequence that directs it from the cytosol to the neuro-secretory vesicle where the tag is (partially) degraded. A neuronal cell line is stably transfected with this construct. Upon depolarization, the targeted luciferase is released into the supernatant together with the neurotransmitter. If the fusion of neuro-secretory cells is inhibited by botulinum toxin, the release of the luciferase is inhibited similar to the inhibition of the release of neurotransmitter, which is responsible for the flaccid paralysis.

ity, are subject to denaturation and inactivation. Therefore, the LD_{50} with respect to the amount of protein fluctuates considerably from batch to batch. Because of the small therapeutic index and potential lethal complication of an overdose, a reliable determination of the biological activity of the BoNT preparation is mandatory (Hakami et al., 2010). The gold standard method to quantify the activity of BoNT is the mouse lethality assay (Pearce et al., 1994, 1997; Singh et al., 2013). This assay determines the dose of the drug that causes asphyxic death in 50% of treated mice. Current estimates are that several hundred thousand mice are killed in this cruel test per year.

Therefore, alternatives to this test are urgently needed and a number of *in vitro* assays have been developed over the recent years. These tests, however, suffer from certain limitations and therefore have not been able to fully replace the mouse lethality assay as yet, although some are already approved for certain applications (Fernández-Salas et al., 2012). A number of approaches were taken. *In vitro* enzyme assays assess the activity of the small subunit in the test tube. These tests fail to test for the activity of the large subunit (Sesardic and Das, 2009). Assays that test the activity of the small subunit within the cell in cell lines that express a tagged substrate protein suffer from low sensitivity (Fernández-Salaz et al., 2007). A number of ELISA based assays were developed that monitor the cleavage of BoNT substrates in cells or synaptosomes (Evans et al., 2009). A recently devised ELISA-based assay that detects a neo-epitope formed after intracellular cleavage of the endogenous substrate protein can substitute for part of the animal experiments used in the quantification of a particular drug, but its use is limited to just one serotype (Fernández-Salas et al., 2012). The assay proposed here holds the promise to overcome these limitations.

The proposed assay is based on a completely different principle. It detects the stimulus-dependent release of a reporter enzyme from neuro-secretory vesicles into the cell culture supernatant as a surrogate for the neurotransmitter release that is inhibited by BoNT (Fig. 1). Neuronal cell lines were stably transfected with plasmids coding for recombinant luciferases that carry an N-terminal tag for redirection into neuro-secretory vesicles. Luciferases are enzymes that convert chemical energy into light by the (ATP-dependent) oxidation of luminogenic organic substrates. The activity of these enzymes can easily be detected in cell culture supernatants after stimulus-dependent release from neuronal cell lines. The new assay tests the activity of both the large and small subunit of BoNT. In addition, it uses an approach that does not involve immunological tools or specific substrates for a particular BoNT serotype but tests the physiological outcome, i.e., the inhibition of neuro-secretory vesicle fusion.

2 Materials and methods

Synthesis of N-terminal tagged luciferase expression constructs

N-terminal tags, selected for directing luciferases into secretory vesicles (Zhang et al., 2009; Bartolomucci et al., 2011; Loh et al., 2002; Taupenot et al., 2003) Chromogranin A (CgA), Secretogranin II (SgII), Carboypeptidase E (CPE) and the leader peptide aa 1-26 of proopiomelanocortin (POMC1-26) were amplified by PCR from human cDNA using proof-reading phusion DNA-polymerase (Thermo-Scientific, St Leon-Rot, Germany), the primers listed in Table 1 and the following protocol: 3 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 60°C and 2 min (POMC1-26: 30 sec) at 72°C. Amplified cDNAs were purified and digested with restriction enzymes: CgA-Luc: hCgA-F/hCgA-R HindIII/NcoI; POMC1-26-Luc and POMC1-26-Ren: hPOMC 1-26-F/hPOMC 1-26-R XhoI/HindIII; CPE-Luc: hCPE-F/hCPE-R XbaI/EcoRI; SgII-NLuc: hSgII-F/hSgII-R KpnI/XhoI; CgA-GLuc: hCgA-F/hCgA-R2 HindIII/KpnI; SgII-GLuc: hSgII-F/hSgII-R2 KpnI/BamHI. Luciferase cDNAs were cut out directly from plasmid pGL3basic (Luc: HindIII/XbaI; CgA-Luc: NcoI/XbaI; POMC1-26-Luc: HindIII/XbaI); pGL4.83 (POMC1-26-Ren: Hind III/XbaI), pNL1.2 (SgII-NLuc: XhoI/BamHI) or pCMV-GLuc-SS (CgA-GLuc: KpnI/XbaI; SgII-GLuc: BamHI/XhoI). Digested tag and luciferase cDNAs were than cloned in-frame into the expression vector pcDNA3. For generation of the cDNA coding for CPE-Luc the firefly-luciferase cDNA was amplified from pGL3basic by PCR with primers Firefly-F/Firefly-R generating recognition sites for EcoRI/ApaI. For generation of the plasmid pcDNA3-hPOMC1-26-GLuc a cDNA coding for the hPOMC1-26-tagged GLuc was amplified from the plasmid pCMV-GLuc-SS with the primer combination hPOMC1-26-GLuc-F/GLuc-R, digested with KpnI/XhoI and cloned in pcDNA3.

Tab. 1: Primer sequences
The primers for amplification of the tag sequences were designed according to the sequence information provided by the accession number given. Italic, overhang for improvement of restriction enzyme cleavage; capital, restriction enzyme recognition site; bold, Kozak sequence.

Primer	Sequence 5' - 3'	Acc. No.	Pos.
hCgA-F	*gcgc*AAGCTT**gccatg**cgctccgccgctgtcct	NM_001275.3	261-280
hCgA-R	*gcgc*CCATGGtgccccgccgtagtgcctgcagc	NM_001275.3	1632-1609
hPOMC 1-26-F	*gcggcg*CTCGAG**ccaccatg**ccgagatcgtgctgcag	NM_001035256.1	264-283
hPOMC 1-26-R	*gcggcg*AAGCTTtgccacgcacttccatggagg	NM_001035256.1	341-321
hCPE-F	*cgcg*TCTAGAa**ccaccatg**gccgggcgaggggcagcgcgct	NM_001873.2	278-303
hCPE-R	*gcggc*GAATTCaaaatttaaagtttctgacatc	NM_001873.2	1706-1684
hSgII-F	*gcggc*GGTA**CCaccatg**gctgaagcaaagacccactg	M25756.1	63-85
hSgII-R	*gcggcg*AGATCTcatattttccattgctctcttag	M25756.1	1913-1891
hCgA-R2	*gcggcg*GGTACCggccccgccgtagtgcctgcagc	NM_001873.2	1706-1684
hSgII-R2	*gcggcg*GGATCCccatattttccattgctctcttag	M25756.1	63-85
hPOMC 1-26-GLuc-F	*gcg*AAGCTT**ccaccatg**ccgagatcgtgctgcagccgctcggg ggccctgttgctggccttgctgcttcaggcctccatggaagtgcgtgg catgaagcccaccgagaacaac	NM_001035256.1	264-283
Firefly-F	*gcggc*GAATTCatggaagacgccaaaaacat		
Firefly-R	*gc*GGGCCCttacacggcgatctttccgccct		
GLuc-R	*gcg*CTCGAGctagtcaccaccggcccccaa		

Generation of stably transfected cell lines
SH-SY5Y (human neuroblastoma), N-2a (murine neuroblastoma), PC-12 (rat pheochromocytoma) and SIMA (human neuroblastoma) cells were cultured in ATCC's recommended medium substituted with 10% (v/v) heat-inactivated FCS, 2 mM stable L-alanyl-L-glutamine and penicillin/streptomycin as antibiotics. For generation of transgenic cell lines cells were transfected with the tag-luciferase expression plasmids by lipofection using Turbofect (Thermo Scientific, St Leon-Rot, Germany). 24 h after transfection cells were treated with growth medium containing 500 U/ml G-418 (Biochrom, Berlin, Germany) for selection of stably transfected cells. Proliferating cell clones were isolated by single cell cloning, expanded and screened for transgene expression by measuring luciferase activity. Cells were lysed in 100 μl passive lysis buffer reagent (Promega, Heidelberg, Germany) and firefly-, renilla- or NanoLuc and Gaussia luciferase activity was measured in 25 μl of cell lysate using the Fluostar Optima (BMG Labtech, Offenburg, Germany).

Density gradient cell fractionation of PC-12 cells and Western blot
PC-12-Luc and PC-12-CgA-Luc cells (5 x 10^7 cells) were re-suspended in homogenization buffer (0.32 M sucrose in 10 mM Hepes pH 7.4 and protease inhibitors) and homogenized in a Potter homogenizer (20 strokes, 3000 rpm/min). The nuclei and cellular debris was pelleted at 750 x g for 5 min at 4°C. The vesicle containing supernatant was layered onto a 0.6-1.8 M linear sucrose gradient and equilibrium sedimentation was achieved by centrifugation in a SW40 rotor (Beckmann) at 30,000 rpm for 5 h at 4°C. Fractions were collected from the bottom (400 μl/fraction). Luciferase activity was measured by adding 10 μl 5x passive lysis buffer to 40 μl of each fraction. For Western blot analysis fractions were diluted ten-fold with 10 mM Hepes pH 7.4 and vesicles were pelleted for 1 h at 100,000 x g. Vesicles were homogenized in 10 mM Hepes pH 7.4 and mixed with Lämmli sample buffer. Proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 5% non-fat dry milk in 20 mM Tris, 136 mM NaCl and 0.1% (v/v) Tween (TBS/Tween) for 1 h at room temperature and incubated with the first antibody (Luc (Promega), CgA (Santa Cruz, USA) and SgII (Thermo scientific) 1:1000) in TBS/Tween containing 5% bovine serum albumin overnight at 4°C and horseradish-peroxidase-conjugated secondary antibody for 2 h at room temperature. Visualization of immune complexes was performed by using chemiluminescence reagent.

Cell fractionation of SIMA cells
SIMA-GLuc- and SIMA-hPOMC1-26-GLuc cells were homogenized in homogenization buffer as described above for PC-12 cells. The crude homogenate was centrifuged at 37,000 x g for 2 h to generate a soluble and vesicular fraction. Vesicles were re-suspended in homogenization buffer and GLuc activity was measured in the soluble or vesicular fractions.

Initial luciferase release from transgenic cells
For release experiments cells were grown in poly-L-lysine treated 96-well plates (5 x 10^4 cells/well) for 24 h. Cells using firefly-, renilla or NLuc-luciferase were then treated with chloroquine-containing growth-medium as indicated. Cells using GLuc as reporter were not treated with chloroquine but were pre-incubated with 100 μl fresh medium for 10 min at 37°C. The medium was aspirated and firefly-, renilla or NLuc-luciferase release was stimulated with 100 μl/well control- (PBS, 137 mM NaCl, 5 mM KCl) or depolarization-buffer (PBS, 5 mM NaCl, 137 mM KCl) for 30 min at 37°C. Release of GLuc was stimulated with 50 μl/well control- (20 mM Hepes pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$ and 1.25 mM $MgSO_4$) or depolarization-buffer (20 mM Hepes pH 7.4, 40.7 mM NaCl, 100 mM KCl, 1.25 mM $CaCl_2$ and 1.25 mM $MgSO_4$) for 3 min at 37°C. The supernatant was transferred and centrifuged at 100 x g for 5 min to remove detached cells. For measuring firefly-, renilla or NLuc-luciferase activity 40 μl of the supernatant was mixed with 10 μl of 5 x luciferase lysis buffer and 50 μl luciferase substrate solution and luciferase activity was determined using a Fluostar Optima. For measuring GLuc activity 10 μl of the supernatant was mixed with 50 μl luciferase substrate solution and measured using a Fluostar Optima.

Optimized luciferase release from BoNT/A treated cells
PC-12-CgA-Luc, SIMA-SgII-NLuc and SIMA-hPOMC1-26-GLuc cells were differentiated with differentiation medium (*PC-12-CgA-Luc*: DMEM supplemented with 1 x B27 supplement, 1 x N2 supplement, 2 mM L-alanyl-L-glutamine, 10 mM Hepes, 1 mM sodium pyruvate, 50 ng/ml NGF and penicillin/streptomycin; *SIMA-SgII-NLuc and SIMA-hPOMC1-26-GLuc*: RPMI supplemented with 1 x B27 supplement, 1 x N2 supplement, 2 mM L-alanyl-L-glutamine, 1 mM non-essential amino acids and penicillin/streptomycin) for 24 h (PC-12-CgA-Luc) or 48 h (SIMA-SgII-NLuc and SIMA-hPOMC1-26). Cells were then incubated with BoNT/A1 (MiproLab, Göttingen, Germany, lot # 3101-AHWW-03_08, activity: 1.4 x 10^8 MLD/mg) in differentiation medium for 48 h. PC-12-CgA-Luc cells were treated with 40 μM chloroquine during the last 24 h and SIMA-SgII-NLuc cells with 50 μM chloroquine during the last 1 h and then the release of luciferase activity into the supernatant was stimulated as described above.

Bacterial firefly luciferase expression and activity
For the expression of firefly-luciferase in bacteria firefly cDNA was cloned from pGL3basic into the bacterial expression vector pRSET and the plasmid was transformed into *E. coli* BL21-pLys cells. Expression of 6-His-tagged-firefly-luciferase was induced with 1 mM IPTG for 5 h and firefly-luciferase was purified from bacteria lysate by Ni-chelate affinity chromatography using Ni-NTA-Superfow matrix (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

For the analysis of firefly-luciferase pH stability, purified firefly-luciferase was incubated with 10 mM Hepes-buffered saline adjusted to pH 3-10 for 2 h at 37°C. After the incubation the pH was readjusted to pH 7 and firefly-luciferase activity was measured.

3 Results

3.1 General concept

A number of proteins are stored together with neurotransmitters in neuro-secretory vesicles. These proteins possess N-terminal leader sequences that target them into the secretory pathway and into the neuro-secretory vesicles. A fraction of these proteins are released from the cell together with the neurotransmitter when the neuro-secretory vesicles fuse with the plasma membrane. It should therefore be possible to target reporter proteins into the neuro-secretory vesicles that can be detected in the cell culture supernatant after stimulation of neurotransmitter release (Fig. 1). Luciferases are commonly used reporter proteins that can be detected with very high sensitivity. Therefore, a number of expression plasmids were generated that code for luciferases

Tab. 2: Expression plasmids

Expression plasmid	N-terminal secretory vesicle Taq	Luciferase
pcDNA3-Luc	none	Firefly
pcDNA3-hCgA-Luc	Human Chromogranin A	Firefly
pcDNA3-hCPE-Luc	Human Carboxypeptidase E	Firefly
pcDNA3-hPOMC 1-26-Luc	Human Proopiomelanocortin aa 1-26	Firefly
pcDNA3-hPOMC 1-26-Ren	Human Proopiomelanocortin aa 1-26	Renilla
pcDNA3-hSgII-NLuc	Human Secretogranin II	Nano
pCMV-GLuc-SS	None	Gaussia
pcDNA3-hSgII-GLuc	Human Secretogranin II	Gaussia
pcDNA3-hSCgA-GLuc	Human Chromogranin A	Gaussia
pcDNA3-hPOMC 1-26-GLuc	Human Proopiomelanocortin aa 1-26	Gaussia

Tab. 3: Depolarization-dependent reporter release in the different cell lines generated upon initial screening
Cell lines were generated as described in the methods section. After establishing monoclonal cell lines, the depolarization-dependent release of luciferase was tested. Where indicated, the test was repeated after optimizing the cell culture conditions, e.g., incubation with chloroquine and/or differentiation into a neuronal phenotype.

Cell line	Reporter construct	Specific reporter release	
		generic	cell line adapted
N-2a (Neuroblastoma Mouse)	Firefly Luc	0.7	2.4
	Chromogranin A – Firefly Luc	0.8	4.8
	Chromogranin A 1..194 – Firefly Luc	0.9	4.7
	POMC 1..26 – Firefly Luc	1.1	2.6
	Carboxypeptidase E – Firefly Luc	1.1	10.0
PC-12 (Pheochromocytoma Rat)	Firefly Luc	1.0	1.4
	Chromogranin A – Firefly Luc	1.5	4.5
	Chromogranin A 1..194 – Firefly Luc	1.5	1.8
	POMC 1..26 – Firefly Luc	2.0	n.d.
	Carboxypeptidase E – Firefly Luc	n.d.	n.d.
	Chromogranin A – Renilla Luc	n.d.	2.6
	POMC 1..26 – Renilla Luc	n.d.	4.2
	Nano-Luc	n.d.	n.d.
	Chromogranin A-Nano-Luc	n.d.	1.5
	Chromogranin A 1..194 –Nano-Luc	n.d.	3.2
	Secretogranin-II-Nano-Luc	n.d.	3.8
SH-SY5Y (Neuroblastoma human)	Firefly Luc	n.d.	n.d.
	Chromogranin A – Firefly Luc	n.d.	2.8
	Chromogranin A 1..194 – Firefly Luc	n.d.	2.2
	POMC 1..26 – Firefly Luc	n.d.	2.9
	Carboxypeptidase E – Firefly Luc	n.d.	3.6
SIMA (Neuroblastoma human)	Firefly Luc	n.d.	n.d.
	Chromogranin A – Firefly Luc	n.d.	1.8
	Chromogranin A 1..194 – Firefly Luc	n.d.	n.d.
	Carboxypeptidase E – Firefly Luc	n.d.	3.2
	POMC 1..26 – Renilla Luc	n.d.	3.3
	Nano-Luc	n.d.	1.3
	Chromogranin A-Nano-Luc	n.d.	1.4
	Chromogranin A 1..194 –Nano-Luc	n.d.	1.7
	Secretogranin-II-Nano-Luc	n.d.	3.1
	Gaussia-Luc	n.d.	1.5
	POMC 1..26 –Gaussia-Luc	n.d.	6.1
	Chromogranin A-Gaussia-Luc	n.d.	4.5
	Secretogranin-II-Gaussia-Luc	n.d.	8.4

that have an N-terminal extension harboring the targeting sequence of different proteins that are normally found in neuro-secretory vesicles (Tab. 2). Different neuronal cell lines were stably transfected with theses plasmids and the stimulus-dependent release of the reporter protein into the cell culture supernatant was determined. In accordance with the general hypothesis, several of these cell lines released the reporter protein after depolarization by increasing the extracellular potassium concentration (Tab. 3). The extent of specific release varied greatly between different cell clones. No systematic correlation between the type of N-terminal target sequence and the extent of specific release was found. Therefore, in an initial attempt to establish a usable cell line, the first PC-12 cell clone stably expressing the firefly luciferase N-terminally tagged with a fragment of the human chromogranine A was further characterized.

3.2 Characterization of firely and nanoluc transfected cell lines

As expected, tagging redirected luciferase into neuro-secretory vesicles. While the luciferase activity of an untagged luciferase was found predominantly on top of the gradient in the fractions that correspond to the cytosol, the majority of the hu-

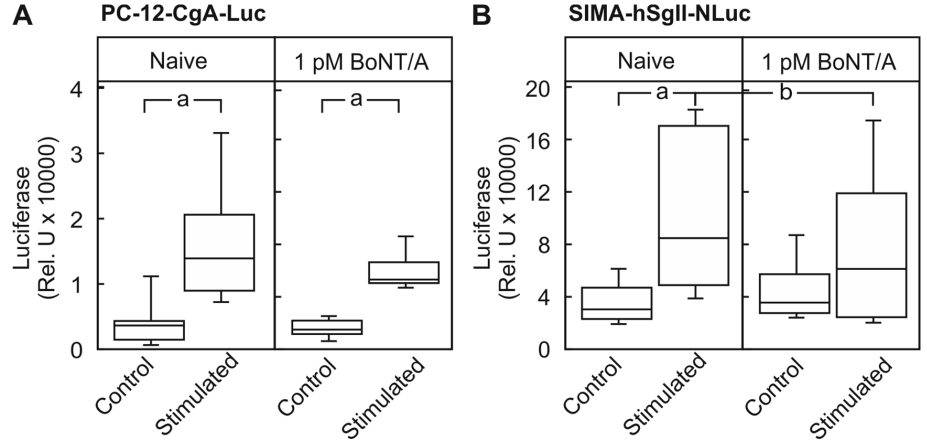

Fig. 2: Inhibition of depolarization-dependent release of luciferase activity from PC-12-CgA-Luc and SIMA-SgII-NLuc cells by BoNT/A

Cells were differentiated with differentiation medium for 24 or 48 h, respectively. Then cells were incubated with 1 pM BoNT/A in differentiation medium for 48 h and 40 μM chloroquine during the last 24 h (PC-12, A) or 50 µM chloroquine during the last 1 h (SIMA, B). Release of firefly luciferase activity was stimulated with control or stimulation buffer for 30 min at 37°C and luciferase activity in the supernatant was measured. Values are medians (bar), upper and lower quartile (box) and extremes (whiskers) of eight independent experiments; values were normally distributed. Statistics: Student's t-test for unpaired samples; a, $p < 0.05$ vs. control; b, $p < 0.05$ vs. naive.

man chromogranine A-tagged luciferase activity was found in denser fractions of the gradient and co-eluted with marker proteins for neuro-secretory vesicles, i.e., secretogranin II and endogenous chromogranine A (not shown). However, the total luciferase activity in cell lysates of cells stably expressing an untagged luciferase was much higher than the total luciferase activity in cell lysates of cells stably expressing the N-terminally tagged luciferase. This was found to be due to the acid instability of luciferase, which lost activity when incubated at low pH. The activity could not be recovered by returning the pH to neutral.

When the acidification of the neuro-secretory vesicles was inhibited by incubating the cells with chloroquine, both total activity and the specifically released luciferase activity were considerably enhanced, resulting in a reliable depolarization-dependent release of luciferase (not shown). When extracellular calcium, which is required for stimulus-dependent fusion of neuro-secretory vesicles with the plasma membrane, was chelated with EGTA, the depolarization-dependent release of tagged luciferase was largely attenuated (not shown).

Since it appeared that the depolarization-dependent release of tagged luciferase from PC-12 cells was dependent on the fusion of the neuro-secretory vesicles with the plasma membrane, it was tested whether the release could be inhibited by prior incubation of the cells with BoNT. Control cells robustly released tagged luciferase in response to depolarization. The specific release appeared to be inhibited by 1 pM BoNT by about 40% (Fig. 2A), however, inter-experimental variations were very large and although a high number of independent experiments were performed, the observed inhibition was not statistically significant.

To overcome this problem, two measures were taken. First, a different neuronal cell line, SIMA, was used because it has been reported that this cell line is particularly sensitive to BoNT/A after differentiation (Fernández-Salas et al., 2012). Secondly, a different luciferase, Nanoluc-luciferase, that has been reported to allow much more sensitive detection than firefly or renilla luciferase, was used as reporter. A cell clone stably expressing the Nanoluc-luciferase with an N-terminal human secretogranin II tag was generated. Upon depolarization with KCl, this cell clone released about 4-times more luciferase than with the NaCl containing control buffer (Fig. 2B). While the unspecific release of luciferase in response to NaCl was not affected by pre-incubation of the cells with BoNT toxin for 48 h, the depolarization-dependent release of luciferase was significantly reduced by 1 pM BoNT. Due to still large inter-experimental variations, it was however not possible to establish a clear dose-response relation in these experiments (not shown).

3.3 Use of an acid-resistant luciferase

The major problem of the experiments with the reporter constructs described so far was the acid lability of the luciferase that necessitated chloroquine treatment of the cells. Although chloroquine treatment increased the depolarization-dependent

release of reporter enzyme in all cell clones, it negatively affected cell viability as judged by the number of cells that detached from the culture plates in the course of treatment. The huge inter-experimental variation most likely reflected random differences in the quantity of cell detachment.

This problem was overcome by the use of a different luciferase, Gaussia luciferase (GLuc). This luciferase is considerably smaller than firefly and renilla luciferases and appears to be more resistant to physical and chemical stresses (Ballou et al., 2000). Thus, in contrast to firefly luciferase, bacterially expressed GLuc retained full activity at a pH of 3.0 over several hours (not shown).

The coding sequence of Gaussia luciferase was extended N-terminally by the leader sequence for the first 26 amino acids of human proopiomelanocortin (hPOMC). SIMA cell clones stably expressing GLuc or hPOMC1-26-GLuc were generated. The leader sequence redirected GLuc in SIMA cells from the cytosolic fraction, in which most of the non-tagged enzyme was detected, to a vesicular fraction (Fig. 3). In contrast to all other cell lines, no treatment with chloroquine was necessary to obtain a reproducible and stable depolarization-induced release of the luciferase from the cells. The specific, depolarization-induced release was about 4 to 5-fold higher than the unspecific release by non-depolarizing buffer (Fig. 4) and was completely abolished by chelation of extracellular calcium with EGTA (Fig. 4).

SIMA-hPOMC1-26-GLuc cells were differentiated into neuronal cells by treatment with N2-/B27 supplement containing medium. These cells were incubated with BoNT for 48 h. In contrast to the other two cell lines described, despite inter-experimental variations, the maximal non-specific release was much lower than the minimal stimulus-dependent release (Fig. 5A). The stimulus-dependent release was significantly inhibited by treatment with 1 pM BoNT. The inhibition was sufficiently robust to establish a dose-response curve (Fig. 5B). Incubation with BoNT resulted in a dose-dependent inhibition of the release of the luciferase into the cell culture supernatant in response to depolarization but did not affect the non-specific release. At a BoNT concentration of 100 pM this inhibition was close to 100%. An EC_{50} of 3.4 pM was determined by non-linear regression analysis (Fig. 5B). To exclude that the inhibition of the stimulus-dependent release by botulinum toxin was due to a non-specific interference with the test system, the experiment was repeated with the highest concentration of BoNT (100 pM) and a corresponding preparation that had been inactivated by heating to 95°C for 20 min. While the active

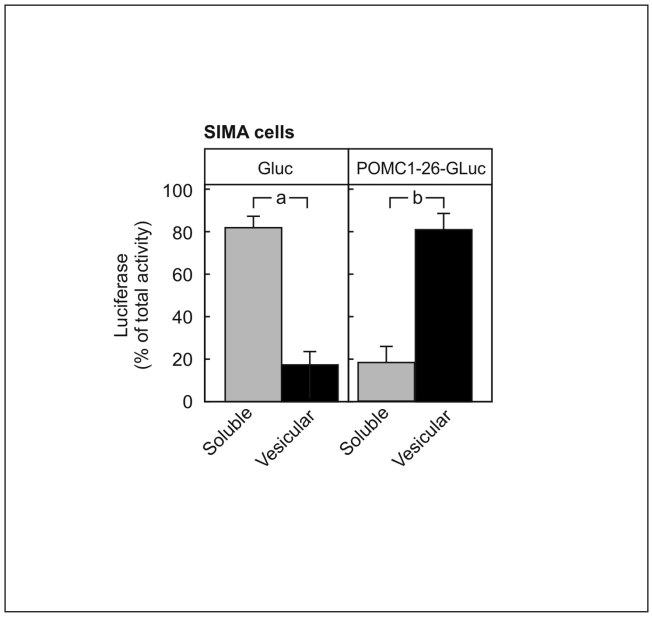

Fig. 3: Subcellular distribution of Gaussia luciferase activity in SIMA-GLuc and SIMA-hPOMC1-26-GLuc cells
SIMA-GLuc and SIMA-hPOMC1-26-GLuc cells were homogenized in an isotonic sucrose/Hepes-buffer. The crude homogenate was centrifuged at 37,000 x g for 2 h to generate a soluble and vesicular fraction. Vesicles were resuspended in homogenization buffer and GLuc activity was measured in the soluble or vesicular fractions. Values are means ± SEM of six measurements in three independent experiments. Statistics: One-way ANOVA with Kruskal-Wallis test; a, $p < 0.05$ vs. vesicular fraction, b, $p < 0.05$ vs. soluble fraction.

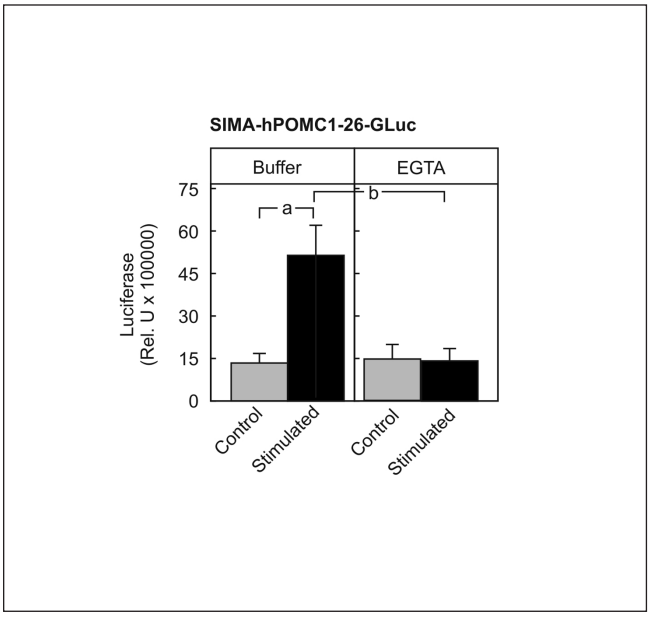

Fig. 4: Inhibition of depolarization-dependent release of luciferase activity from SIMA-hPOMC1-26-GLuc cells by the Ca^{2+}-chelator EGTA
Cells were pre-incubated with fresh medium for 10 min at 37°C. Release of Gaussia luciferase activity was then stimulated with control (Na^+-HBS: 136 mM NaCl, 4.7 mM KCl), or stimulation buffer (K^+-HBS: 40.7 mM NaCl, 100 mM KCl) in the presence or absence of 10 mM EGTA for 3 min at 37°C and luciferase activity in the supernatant was measured. Values are means ± SEM of 11 to 12 measurements in three independent experiments; values were normally distributed. Statistics: Student's t-test for unpaired samples; a, $p < 0.05$ vs. buffer-control; b, $p < 0.05$ vs. buffer.

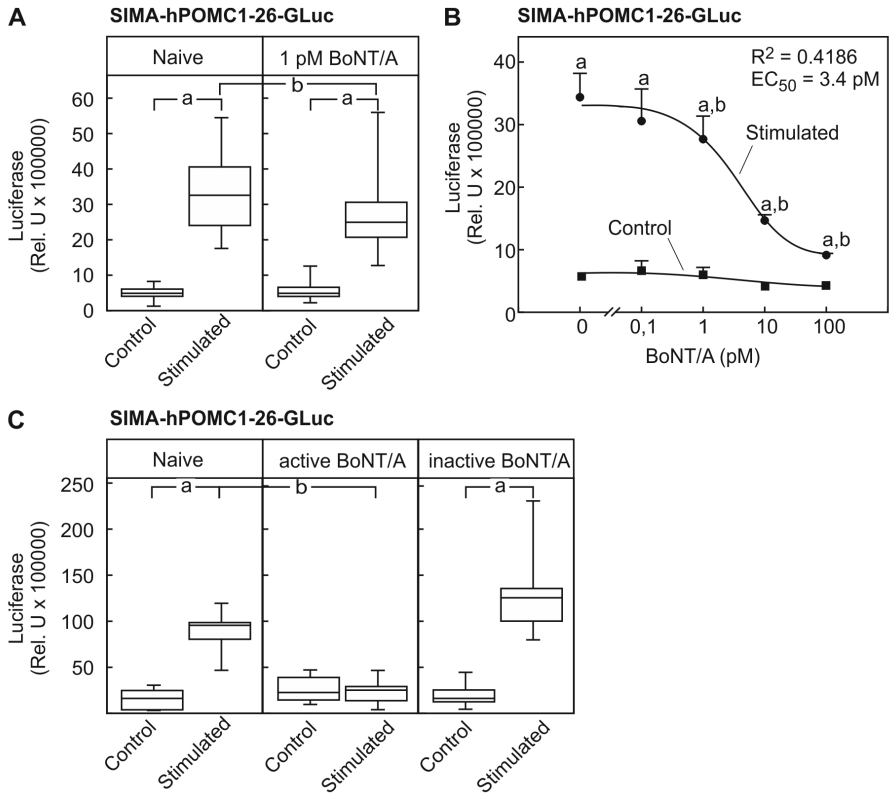

Fig. 5: Inhibition of depolarization-dependent release of luciferase activity from SIMA-hPOMC1-26-GLuc cells by BoNT/A
SIMA-hPOMC1-26-GLuc cells were differentiated with differentiation medium for 48 h. (A) Then cells were incubated with 1 pM BoNT/A in differentiation medium for 48 h. Release of Gaussia luciferase activity was stimulated with control or stimulation buffer for 3 min at 37°C and luciferase activity in the supernatant was measured. Values are medians (bar), upper and lower quartile (box) and extremes of ten independent experiments; values were normally distributed. (B) Cells were incubated with 0.1-100 pM BoNT/A in differentiation medium for 48 h. Release of Gaussia luciferase activity was determined as in A. Values are means ± SEM of 10 (0 to 1 pM) or 6 (10 and 100 pM) independent experiments. The dose response curve was determined by non-linear regression analysis of the individual values of all experiments. (C) In a different set of experiments cells were incubated with 100 pM active or heat-inactivated botulinum toxin and the release of Gaussia luciferase was determined as above. Values are medians (bar), upper and lower quartile (box) and extremes (whiskers) of 11 to 16 measurements in three to four independent experiments; values were normally distributed. Statistics: Student's t-test for paired samples; a, $p < 0.05$ vs. control; b, $p < 0.05$ vs. naive.

BoNT abolished the stimulus-dependent release of the reporter enzyme almost completely (Fig. 5C), the stimulus-dependent release was not affected by the heat-inactivated toxin, precluding any non-specific interference of the preparation with the test system.

4 Discussion

The current study provides the proof of principle that luciferases that are extended by N-terminal leader sequences of proteins normally sorted into neuro-secretory vesicles are redirected into neuro-secretory vesicles and can be used to monitor depolarization-dependent fusion of these vesicles with the plasma membrane. Neuronal cell lines expressing these proteins can be used to sensitively detect the inhibition of depolarization-dependent reporter release by prior incubation with BoNT and may serve as an alternative method to quantify BoNT activity in replacement of the currently widely used mouse lethality assay.

The assay for BoNT described here offers a number of advantages over other *in vitro* approaches. Many *in vitro* approaches only test the activity of the enzyme activity of the small subunit of BoNT. Thus, a number of substrates have been designed that allow monitoring the proteolytic activity with high sensitivity. These assays have the great advantage that they are robust, fast biochemical assays that involve no biological material. Thus, recently an assay was described in which the FRET in a double CFP and YFP-labeled SNAP25

or Synaptobrevin molecule was disrupted by BoNT cleavage of the central part of the molecule connecting the two fretting fluorescent proteins (Joshi, 2012; Sapsford et al., 2011). The inherent disadvantages of tests like this are (1) that they do not provide any information about the activity of the large subunit of BoNT and (2) that a special test is needed for every botulinum toxin serotype. The former problem can be overcome by introducing the probes into cell lines and by measuring the change in FRET *in vivo* in cell culture. Such an assay procedure has been described, however, its sensitivity was very low (Dong et al., 2004).

Similarly, by reconstituting the receptors for the large subunit into liposomes that contain a fluorescence quenched substrate in their lumen, both the activity of the large subunit and the small subunit could be tested in a complete *in vitro* system. Because the substrate is serotype-specific, this assay only can be used for one specific serotype.

A very sensitive different approach was taken by Fernández-Salaz et al. (2012) who developed an assay that is currently used by Allergan company as an accepted alternative method for the LD50 assay in mice. In this assay, the generation of a neo-epitope as a result of the cleavage of the endogenous SNAP25 by BoNT in SIMA cells was detected with a monoclonal antibody based ELISA. This assay tests both the activity of the large and small subunit of BoNT but still has the disadvantage that it is applicable for only one serotype of BoNT that targets SNAP-25. Although the assay described here was performed with only one BoNT serotype, in theory it should be equally applicable to other serotypes because not the cleavage of a particular substrate was determined but rather an event downstream of this cleavage, i.e., the fusion of the neuro-secretory vesicles, was monitored. An additional advantage is that while low expression levels of the SNARE proteins cleaved by the different BoNT serotypes poses a problem to the immunological techniques that detect their cleavage products, it rather is an advantage in the functional test system described here because the sensitivity will increase as the amount of cellular BoNT substrate, and hence the functional reserve that still allows vesicle fusion, decreases.

Only one currently devised *in vitro* assay shares these properties: A spinal cord skeletal muscle co-culture system (Eckle et al., 2014). In this assay, fetal spinal cord cells and skeletal muscle cells are differentiated in a co-culture system over a period of three weeks and subsequently incubated with BoNT. In these cultures spontaneous contraction of skeletal muscle cells is monitored by automated video analysis. The authors showed that this activity was inhibited by BoNT already in concentrations as low as 50 fM. In comparison to the assay presented in this study, the co-culture suffers from an almost prohibitive technical complexity that precludes wider use and it still relies on the use of experimental animals, since it is based on primary cell cultures generated from fetal tissue.

The assay presented here uses a human derived cell line. This might be an advantage since it has previously been shown that, depending on the BoNT serotype, large inter-species differences exist in the sensitivity towards the toxin (Pellett, 2013; Whitemarsh et al., 2013).

The sensitivity of the new assay lies in a range that is suitable for testing pharmacological preparations. Thus, the lowest dose of BoNT that caused a statistically significant inhibition of the depolarization-dependent reporter release (1 pM, Fig. 5) roughly corresponds to 2.1 mouse LD_{50} units. A normal mouse lethality assay covers a range of 0.6 to 2.5 mouse LD_{50} units. The lowest dose detected in this assay thus lies in the dose range normally tested in the mouse lethality assay.

By using the extremely acid stable Gaussia luciferase whose activity is not affected by the low pH in neuro-secretory vesicles, the use of chloroquine to stabilize the tagged vesicular luciferase could be omitted. This helped to augment the difference between non-specific and specific reporter release and to decrease inter-experimental variation. Still, additional improvements are needed to further reduce this variation and to increase the reliability of the test.

Since heat-inactivated BoNT did not inhibit stimulus-dependent reporter release a non-specific interference of the BoNT-preparation with the assay could be excluded. To further prove the dependence on the activity of either BoNT subunit, experiments with subunit-specific neutralizing antibodies might be useful. It is important to note that the assay thus far was only performed with purified BoNT. Pharmacological preparations contain a number of additives, for example to increase stability and shelf life. It cannot be excluded at the current stage that such additives might interfere with the assay described.

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Conflict of interest

No conflicts of interest to declare.

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