Split luciferase-based assay to detect botulinum neurotoxins using human motor neurons derived from induced pluripotent stem cell.

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Abstract

Botulinum neurotoxins (BoNTs) have been widely used clinically as a muscle relaxant. These toxins target motor neurons and cleave proteins essential for neurotransmitter release like Synaptosomal-associated protein of 25 kDa (SNAP-25). Most *in vitro* assays for BoNT testing use rodent cells or immortalized cell lines, which showed limitations in accuracy and physiological relevance. Here, we report a cell-based assay for detecting SNAP25-cleaving BoNTs by combining human induced Pluripotent Stem Cells (hiPSC)-derived motor neurons and a luminescent detection system based on split nanoluciferase. This assay is convenient, rapid, free-of-specialized antibodies, and can discriminate the potency of different BoNTs, with a detection sensitivity of femtomolar concentrations of toxin and can be used to study the different steps of BoNT intoxication.

Abbreviations: BoNT, Botulinum neurotoxin, SNAP-25, Synaptosomal-associated protein of 25 kDa, hiPSC, human induced Pluripotent Stem Cells, SNARE, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor, SV2, synaptic vesicle proteins, MLB, mouse lethality bioassay, LD₅₀, toxin's dose lethal for half of the animal injected, CB-assay, cell-based assays, FRET, Förster resonance energy transfer, Concanamycin A, EC50, Half maximal effective concentration, MNs, motor neurons.

Introduction

Botulinum neurotoxins (BoNTs) are a family of bacterial toxins produced by the bacteria *Clostridium botulinum*. Seven serotypes (named A to G) can be distinguished based on their immunological differences [1] and further divided in subtypes based on their amino acid sequence differences [2]. Recently, a genomic database search revealed additional BoNT-like toxins like BoNT/X and BoNT/En [3] [4]. Botulinum toxins target peripheral motor neurons and block the release of acetylcholine by cleaving members of the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) complex, essential for neurotransmitter release. This leads to muscular relaxation and flaccid paralysis [5]. BoNTs are used in the clinic to treat muscle dysfunction and an increasing number of other indications [6]. The toxin is produced as a single polypeptide chain of 150 kDa which is post translationally cleaved. This yields a di-chain consisting of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) linked by a disulfide bond.

The intoxication mechanism involves at least four steps: receptor binding by the HC, HC and LC internalization in endosomes, LC translocation into the cytosol and finally specifically cleavage of neuronal SNARE proteins by the LC [7].

More precisely, the HC binds selectively to a receptor complex at the presynaptic surface. The binding is initiated by a neuron-specific ganglioside such as GT1b and GD1a [6] followed by the binding to a neuronal protein receptor such as synaptic vesicle proteins SV2 and Synaptotagmin [7] [9] [10] [11]. This complex is then internalized into the cell by endocytosis. After acidification of the endosomal vesicle, the

LC is translocated into the cytoplasm and the endopeptidase domain of the LC interacts and cleaves its substrate.

Because BoNTs have a biological origin and are highly toxic, the safe and effective dosing of pharmaceuticals BoNT requires accurate and reliable test. For years, the gold standard assay to test BoNT pharmaceuticals has been the mouse lethality bioassay (MLB) [12]. This assay measures the toxin's dose lethal for half of the animal injected (LD_{50}). This method causes severe suffering of laboratory animals and therefore doesn't follow the principle of the 3Rs (reduction, replacement and refinement of animal use in research). Therefore, the development of alternative methods is socially and ethically required.

Several *in vitro* cell-based assays (CB-assay) have been developed for BoNT potency. The main advantage of these CB-assays is that they can provide information on all essential steps of BoNT intoxication. Several methods were approved by the FDA and other regulatory agencies, to test the potency of pharmaceutical BoNT/A [13] [14] [15]. All these assays monitor the cleavage of BoNT substrate.

The first replacement assay was developed by Fernandez *et al.* and relies on a monoclonal antibody that recognizes only the cleaved form of the synaptosomal-associated protein of 25 kDa (SNAP-25), a component of the neurotransmitter release apparatus. It was developed in human SiMa cell line in an ELISA-based format [13]. Another test developed by Dong *et al.* in a rodent cell line (PC12), uses recombinant Förster resonance energy transfer (FRET). Two fluorescent proteins (CFP and YFP) enabling FRET are linked together with a SNARE protein fragment. The proteolytic activity of BoNT abolishes energy transfer between the fluorescent proteins [15].

Rodent cells and immortalized cell lines have limitations concerning accuracy and physiological relevance to the responses observed in humans. Human induced Pluripotent Stem Cells (hiPSC)-derived neurons could serve as a useful cell model to improve these assays. Several studies have shown that these cells are a highly sensitive model to test BoNT, especially hiPSC derived motor neurons (MNs) that represent the most physiological system to study BoNT [16] [17] [18] [19].

In this work we describe the development of a new cell-based assay combining human iPSC-derived MNs with an antibody-free detection system uses the engineered split-Nanoluc luciferase reporter (NanoBiT), [20] [21] to detect toxin action. We developed a toxin sensor constituting of a single chain polypeptides composed of two complementary fragments of Nanoluc luciferase linked through substrates of BoNT. In presence of toxin, the linker region is cleaved, and the two luciferase pieces are separated resulting in decrease of luminescence emission.

The assay presented here offers the possibility to test toxins after an acceptable timeframe of cell culture (9 days) in physiologically relevant cells and can be read directly after toxin treatment. Its sensitivity allows to detect functionally active toxin in the femtomolar range, to compare and rank

potency of different BoNTs. In addition, we demonstrate that our assay can be used to evaluate not only the process of target cleavage, but also toxin binding and translocation.

Material And Methods

Plasmid

For BoNT/A acellular and rodent cells detection, the construct was cloned into pET28a vector with *NocI/NotI* and the inserts were Nnano-SV2C(529-566)-G4S-SNAP25(141-206)-Cnano and Nnano-SNAP25(141-206)-Cnano. Split nanoluc sequence was kindly provided by Promega [21]. Overlap PCR was used to obtain full inserts and then ligation of insert with cutted vector. For BoNT/B *in vitro* detection, the construct was Nnano-VAMP(35-96)-Cnano.

For BoNT/A detection in neurons, the construct was designed using split Nanoluc, then firefly as internal controls. The internal control is located in front of SNAP25 (Full length). The vector was based on dual hSyn promotor vector [22] and *BamHI/NotI* were used for cloning. This cellular sensor is named here “BoNT sensor4”.

Lentiviral vector production

High-titer (1.3×10^9 TU/ml) viruses were produced by Neo Biotech (Clinisciences, Nanterre, France) using the dual promotor vector expressing the polypeptide BoNT sensor4 as lentiviral expression vector.

His tag sensor proteins purification

Different constructs of sensor proteins were purified using IMAC. The His6 tag was cloned into pET28a vector at C terminal. BL21 containing correct plasmid of *in vitro* construct was inoculated overnight and cultivated at 37°C until O.D. around 0.6-0.8, then induced by 0.25 mM of IPTG (final concentration) for overnight at 20 °C. The cells are harvested by centrifuging at 4500g, 10 minutes. After that, cells pellets were dissolve in 50 mM HEPES, 150 mM NaCl, pH7.4 buffer and followed by sonication for 3 minutes.

The supernatant of cell lysate was saved and mixed with Ni²⁺ beads for 1 h with rotation in 4°C. Later, the mixture was loaded into column and the resin was washed with 10 fold of bed column of 20 mM Imidazole in 50 mM HEPES, 150 mM NaCl, pH7.4 buffer. Finally, the protein was eluted in 4 fold of bed column 500 mM Imidazole in 50 mM HEPES, 150 mM NaCl, pH7.4 buffer. The purified sensor protein was dialyzed into 50 mM HEPES, pH 7.1 buffer to perform the assay immediately.

In vitro detection assay of Botulinum toxin

Sensor protein concentration was estimated by SDS-PAGE with standard BSA as reference. 30 nM of sensor protein was prepared in 50 mM HEPES, 20 μ M ZnCl₂, 2 mM DTT, pH 7.1 buffer. The Botulinum toxin A was diluted and added into sensor protein solution with a concentration series from 1 nM to 1 fM with dilution factor 10. After 4 h and 24 h incubation at 37°C, Nano-Glo susbrate (Promega) was added into each sample with equal volume. The luminescent signal was measured in plate reader. Assay was

performed duplicate. Negative controls were also designed and performed. BoNT/A was mixed with Nnano-VAMP-Cnano sensor protein or BoNT/B was mixed with Nnano-SV2C-p25-Cnano sensor protein.

Rodent detection assay of Botulinum toxin

Lentivirus particles expressing the polypeptide BoNT sensor4 was added into 7-days neuron cells and then Botulinum toxin A of a series concentration from 300 pM to 1 pM was added into 13-day neuron cells with triplicate. After 48h challenging to toxin, the medium was removed, and a luciferase assay was done as described below.

hiPSC-derived Motor Neurons culture

Frozen iCell MNs provided by FujiFilm Cellular Dynamics International (FCDI, Madison, WI, USA) were thawed and plated according to the provider instructions at the recommended density (100×10^3 cells/cm²) into 96-well plates (TPP, Trasadingen, Switzerland or Greiner Bio-One International GmbH, Kremsmünster, Austria) in a volume of 100µl per well. Cells were maintained in the medium recommended at 37°C, 5% CO₂ for the indicated time. A 50% to 75% medium change was realized every 2 to 3 days as recommended by suppliers. Cultures were observed regularly using an Olympus microscope (CKX53, Olympus Life Science Solutions, Waltham, MA, USA).

Immunostaining

Eight days after plating, iCell MNs were fixed with 4% paraformaldehyde for 15 min at RT. Cultures were washed twice with PBS (Thermo Fisher Scientific) and then permeabilized and blocked with PBS containing 2% BSA (Sigma-aldrich, Saint-Louis, MI, USA) and 0.1% triton (X100, Sigma-aldrich) for at least 1h at RT. Primary antibody was incubated overnight at 4°C in PBS 2% BSA, 0.1% triton. Cultures were then washed with PBS and stained with the corresponding secondary antibodies and DAPI for 1h at RT and then washed with PBS. Primary antibodies used in this study are: Chicken Anti-Tubulin (Ab107216, Abcam Cambridge, United Kingdom; 1:1000), Goat Anti-Islet1 (GT15051, Neuromics Inc., Edina, MN, USA; 1:200). Secondary antibodies, Alexa 647 Donkey anti-Goat (A21447, Thermo Fisher Scientific), Alexa 594 Goat anti-Chicken (A11042, Thermo Fisher Scientific), were used at 1:1000 dilution. Finally, cell cultures were washed in PBS.

Image acquisition

Images were acquired at room temperature using the ImageXpress Micro Confocal High-Content Imaging System (Molecular Devices, San José, CA, USA) at 10X and 20X magnification. Images were analyzed in MetaXpress software (Molecular Devices). A multi wavelength cell scoring analysis was used to count DAPI⁺ and ISLET⁺ nucleus. Finally, pictures were saved in TIFF format and compiled into figures in Illustrator (Adobe).

Botulinum Neurotoxins

For acellular assays and rodent CB-assay, BoNTs were purchased from Metabionics (Madison, WI, USA).

Recombinant research grade botulinum neurotoxins A and E were manufactured from *E.Coli* by Ipsen as previously described [23] [24] [25]. Inactive (endonegative) BoNT/A (BoNT/A(0)) was produced by point mutations E224Q, H227Y in BoNT/A [25]. All BoNTs were purified and activated to more than 91% final purity. For assessing the effect of BoNT serotypes A and E, hiPSC-derived neurons were plated in 96-well plate and exposed to the indicated doses of BoNT/A or BoNT/E in 100 µl of culture medium. For each experiment, each dose of BoNT was tested in triplicate. A negative control (medium without toxin) was always included.

hiPSC-derived Motor Neurons detection assay of Botulinum toxin

48 h after plating, iCell MN were transduced with lentivirus particles expressing the polypeptide BoNT sensor4, at MOI of 16, in 40 µl of culture media. 4-6 h after, the media was removed, and 100 µl of fresh medium was added. After 8 days of culture, cells were treated with BoNTs for 24 h, as described above, after which time the medium containing the toxin was removed and a luciferase assay was done as described below.

Western Blot

All samples were resolved by SDS-PAGE (Invitrogen, Carlsbad, CA, USA) and transferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat milk in 0.1% PBS-T (phosphate buffered saline with Tween-20) and probed with an anti-Firefly antibody (ab185924, Abcam, 1/5000) or anti-SNAP25 antibody (S9684, Sigma-Aldrich, 1/2000). Immunoreactive bands were detected using horseradish peroxidase–conjugated secondary anti-rabbit (A6154, Sigma-Aldrich, 1/2000) antibodies and SuperSignal West Dura ECL substrate (Thermo Fisher Scientific). Bands were visualized on a Pxi4 imaging system using GeneSys image acquisition software (Syngene, Bangalore, Karnataka, India).

For SNAP-25 cleavage assay, intensities of the total form and cleaved form of SNAP25 were measured with GeneTools (Philomath, OR, USA).

GT1b assay

Six days after thawing, GT1b (30µM) was added to the media recommended by supplier of previously transduced iCell MNs. 48h later, this media was removed, and the cells were treated with rBoNT/A (1pM). After 24h the medium containing the toxin was removed and a luciferase assay was done as described below.

Concanamycin A (ConA) Assay

Eight days after thawing, transduced iCell MNs were treated with rBoNT/A (1nM) in medium recommended by supplier supplemented with 60 mM KCl for 10 min. Toxins were then washed off using

PBS. Where appropriate, ConA (250nM, Sigma-Aldrich) was added to the culture. 4h later, a luciferase assay was done as described below.

Luciferase assay

For rodent neurons assay, cells were lysed by adding 200 µl passive lysis buffer (Promega) and incubated at room temperature for 20 minutes. 50 µl of cell lysate was mixed with 50µl firefly luciferase substrate (ONE-Glo(TM) EX reagent) and measured. Subsequently, 50µl Nanoluc luciferase substrate (NanoDLR Stop & Glo(R) reagent) and measure it again.

For hiPSCs derived neurons assay, after cell culture medium removing, 80 ml of fresh DMEM w/o phenol red was added. The cells were incubated for 30 min at RT.

Luciferase activity (NANOLUC™ and Firefly luciferase) was assayed using the Nano-Glo[®] Dual-luciferase Reporter kit accordingly to the manufacturer's protocols (Promega) and using the multimode plate reader VICTOR[®] Nivo™ (Perkin Elmer, Waltham, MA, USA). With this protocol, luminescence was measured in less than one hour following toxin treatment.

For each cell lysate sample, both Firefly and NANOLUC™ luciferase signals were measured and the "luminescence ratio" (NANOLUC™ / Firefly = R_i) was calculated.

Each sample's ratio R_i was normalized with the mean ratio of sample without toxin exposure.

For in vitro and rodent experiment, data are expressed as remaining sensor activity, as follows:

- Ratio percentage = $100 * (R_i / R_{w/o \text{ toxin}})$

For the human motor neurons data, results are expressed as a percentage of toxin activity, as follows:

- % activity = $100 * [1 - ((R_i - R_{w \text{ toxin MAX}}) / (R_{w/o \text{ toxin}} - R_{w \text{ toxin MAX}}))]$ where $R_{w/o \text{ toxin}}$ is the mean ratio of sample without toxin exposure and $R_{w \text{ toxin MAX}}$ is the mean ratio of sample with the highest dose of toxin.

Finally, for one specific toxin concentration, BoNT sensor4 cleavage percentage is determine as follows:

- BoNT sensor4 cleavage (%) = $100 * [1 - (R_i / R_{w/o \text{ toxin}})]$

Results shown are from four independent experiments in triplicates.

Statistics

All results are presented as mean $\pm$ SEM of n independent experiments. Dose-response curves were fitted with a four-parameter logistic equation, and the pEC50 was calculated. All data processing and statistical tests were done using GraphPad Prism version 8 (GraphPad Software Inc., La Jolla, CA, USA). Statistical

analysis indicates one or two-tailed t-test or ordinary one-way ANOVA multiple comparisons P values, as indicated in figure legends. Significance is indicated by asterisks or NS, as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; **** $P < 0.0001$; NS: not significant $P > 0.05$.

Results

Development of an assay based on a split form of Nanoluc luciferase to detect BoNT activity.

In order to establish a novel cell-based approach to detect the activity of BoNT, we first developed an antibody-free detection system using recently developed split-Nanoluc luciferase [21]. More precisely, we expressed a single polypeptides chain composed of Nanoluc luciferase split in two parts and maintained close to each other by a linker containing BoNT substrates sequence. Once BoNT cleaves its target, the two parts of the Nanoluc luciferase are no longer in proximity, resulting in a decrease in its activity (**Figure 1A**). The remaining luciferase activity can be inversely correlated with the toxin activity.

Several single chain polypeptides containing different BoNT substrates were designed (**Figure 1B**). For instance, a sensor contains a linker composed of a SNAP-25 fragment that can be cleaved by BoNT/A, E and C and another sensor contains an additional fragment of SV2C, functioning as toxin receptor, in addition to the SNAP-25 fragment. The third version uses a VAMP 1 fragment as the linker, which can be cleaved by BoNT/B, D, F and G. These sensors proteins were purified as recombinant proteins.

Then, to evaluate the functionality of these sensor proteins and their respective sensitivity to BoNT, the three version of sensors were incubated with a gradient of concentration of BoNT/A or BoNT/B for 24 hours and the remaining luciferase activity was measured.

Both version of sensors containing a SNAP-25 fragment showed a concentration-dependant decrease of their luciferase activity after BoNT/A incubation (**Figure 1C**). Moreover, the sensor that contains SV2 fragment has an estimated EC₅₀ at 7.86 pM. This is approximatively 5-fold more sensitive than the sensor without SV2C (EC₅₀=35.87pM), indicating that including fragment of toxin receptor can improve the sensitivity of the sensor (**Figure 1C**). BoNT/B did not affect the luciferase activity of these two sensors, demonstrating the specificity of these two sensors for BoNT/A (**Figure 1C**).

The third sensor showed a concentration-dependant decrease of their luciferase activity after BoNT/B incubation (**Figure 1D**). Incubation of this sensor with BoNT/A did not affect the luciferase activity, demonstrating its specificity for BoNT B (**Figure 1D**).

All these data show that the split-Nanoluc luciferase-based sensors can be used to detect BoNT activity.

NANOLUCTM sensor can detect BoNT/A in cells and human motor neurons

We next sought to develop cell-based assays using split-Nanoluc luciferase-based sensors. As the majority of BoNT products are BoNT/A, we focused on establishing a SNAP25-cleaving cell-based sensor. For this purpose, a construct expressing a split-Nanoluc luciferase with a linker containing a firefly

luciferase, which serves as an internal control, and the full length of SNAP-25 was developed. This recombinant reporter was named BoNT sensor4 and it is illustrated in **Supplementary Figure 1**. Then, this sensor protein was expressed in neurons via lentiviral particles, as developed by Gascon and co-workers [22] and adapted by us for co-expressing BoNT sensor4 and GFP. The lentiviral expression vector is illustrated in **Figure 2A**.

Primary rat cortical neurons transduced with these lentiviral particles were exposed to various concentrations of BoNT/A and cell lysates were harvested 48 hours later. The luciferase activities of both firefly and NANOLUC™ luciferase were measured. Incubating neurons with toxin reduced the signal of NANOLUC™ luciferase, showing BoNT sensor4 ability to sense toxin activity in cells (**Figure 2B**).

We next decided to transfer this assay into a well characterized, commercially available hiPSC model, the iCell Motor neurons. This hiPSC model has been shown to be highly sensitive to BoNT serotypes A and E [26], and to express, after 14 days of culture, markers of authentic and functional motor neurons [19]. First, we checked the biosensor expression 14 days after thawing, using lentiviral particles. We transduced iCell MNs and after two weeks of culture we observed that the majority of the MNs were GFP-positive (**Supplementary Figure 2**), indicating that lentiviral particles are efficient in transducing these human cells. After cell lysis we checked the expression of the engineered polypeptide by Western blot using an antibody against Firefly luciferase. This blot revealed a single band at the expected molecular weight suggesting that the SNAP-25 sensor is well expressed (**Supplementary Figure 2**).

Because our previous work strongly suggested that 7 days of maturation were enough for iCell MNs to express motoneuronal markers and proteins involved in BoNT intoxication [19], we compared toxin sensitivity after 1 or 2 weeks of culture. At these two different stages of maturation, we treated cells with BoNT/A for 24 hours and we tested SNAP-25 cleavage by western blot. At the two time points tested, we observed a concentration-dependent increase in SNAP-25 cleavage with a complete cleavage of the target at higher concentrations. The two curves were similar with a comparable pEC50 (-11.89 ± 0.03 at day 7 and -11.89 ± 0.04 at day 14-15) indicating a similar sensitivity to the toxin (**Figure 2C**). This result indicates that the extra week in culture did not change toxin-sensitivity of MNs.

Finally, we checked, at an early maturation time point, if transduction disturbed cell identity of MNs. After 8 days of culture, we fixed transduced cells and quantified MNs using the nuclear motoneuronal marker ISLET1. **Figure 2D, 2E and supplementary figure 3** show that, at this stage, the number of ISLET⁺ cells per well in transduced conditions were similar to the control. Therefore, no negative impact on the motoneuronal identity of the cell culture was detected, showing that transduction does not impair motor neurons differentiation.

Altogether these results confirm that iCell MN overexpress BoNT sensor4 and already after 7 days in culture can be used for a cell-based assay without losing toxin sensitivity.

Femtomolar concentrations of BoNT/A or /E can be measured in human MNs expressing NANOLUC™ sensor less than one hour after toxin treatment end.

Based on these results we established a new CB-assay protocol that can be run in 9 days, as summarized in **Figure 3A**. Briefly, iCell MNs are transduced 48 hours after plating, followed by 6 days of further cell culture. Then, a 24 h toxin treatment 8 days after first plating is done, after which luminescence signals can be directly measured.

To evaluate the sensitivity of this new CB-assay, we established concentration-response curves for two different recombinant BoNT serotypes, BoNT/A and BoNT/E. Both toxins showed a concentration-dependant increase of toxin activity (**Figure 3B**). Maximal activity resulted in complete cleavage of SNAP-25 and was observed at the highest concentration for both toxins. As shown by us previously using a western blot SNAP-25 cleavage assay [19], BoNT/E ($pEC_{50}=13.57\pm0.05$) was significantly more potent than BoNT/A ($pEC_{50}=12.57\pm0.05$) in this new assay (**Figure 3C**). As a control, a mutant form of BoNT/A with inactivated LC did not lead to any changes in luminescence.

Mechanistic question can be addressed by the new CB-assay

To determine if the new CB-assay could also be used to study the mode of action of BoNT, we evaluated the effect of two well-known modulators of BoNT binding and LC translocation.

It was previously shown that addition of ganglioside GT1b, a BoNT coreceptor, could improve toxin sensitivity of hiPSC derived neurons [14] by facilitating BoNT binding. To evaluate if our new CB-assay would be sensitive to gangliosides, we added 1 pM of GT1b to the cell culture, 48 hours before treatment with BoNT/A. **Figure 4A** shows that addition of GT1b increased the cleavage of the engineered target.

Moreover, using ConcanamycinA (ConA), a specific vacuolar H^+ -ATPase inhibitor, which blocks endosomal acidification and therefore antagonized the action of BoNT [27], we tested if our assay is sensitive to this effect. **Figure 4B** shows that treating transduced MNs with 1 nM BoNT/A in high- K^+ concentration conditions (to stimulate synaptic vesicles), for 10 min, resulted in approximately 30% substrate cleavage during a subsequent 4 hours. This effect was completely blocked when 250 nM of ConA was co-administrated with the toxin to cells.

Together, our data show that the new cell-based assay described here reports on all intoxication steps by botulinum toxins and therefore can also be used for mechanistic studies.

Discussion

In this paper, we describe a new cell-based assay that provides a reproducible and highly sensitive model for SNAP-25 cleaving toxins and it is independent from using any specialized antibodies. Moreover, the assay combines human iPSC derived MNs, which represent a physiological cellular model for BoNT activity, with a straightforward, simple detection of the enzymatic action of the toxin activity by directly quantifying luminescence after toxin treatment.

BoNT testing by the new assay is a further development of previous work. Several studies already demonstrated the potential of hiPSC-derived MNs for BoNT testing [17] [18] [19]. More precisely, it has been shown that these cells are a sensitive neuron type for BoNTs [17] and express marker proteins of mature motor neurons [19].

Previous studies also suggested that the time in culture of iCell MNs, before BoNT testing, could be shortened [16][19]. If 14 days are required to obtain highly mature and functional MNs, we confirmed here that we could test the toxin from day 7 without losing sensitivity.

Moreover, the “direct” luminescent read-out used here, also enabled to measure BoNT activity less than one hour after toxin treatment. Compared to a cell-based assay with an ELISA read out [13], the assay described here does not need several steps of incubation and wash due to the use of antibodies. Furthermore, it doesn't require any special antibody which can be costly and difficult to produce. Overall, our assay is cheaper, easier and faster than an ELISA-based readout.

While alternative assays have been developed with fluorescent probes in an effort to avoid using antibodies [15], the major drawback of these assays required complicated equipment such as fluorescent microscope. Alternative readouts based on natural luminescence [28] or on electrochemical multi-electrode [29] arrays have been developed. For example, Pathe-Neuschafer-Rube and co-workers developed a pH-stable firefly-luciferase targeted to secretory vesicles in the human SiMa cell line and showed that BoNT/A could inhibit luciferase secretion [28]. Such approaches do circumvent a limitation of our assay which is to work theoretically for all BoNT serotypes. Indeed, we designed a specific SNAP25-cleaving BoNT assay, but our system is very versatile and BoNT target sequence can be easily changed. The production of *in vitro* sensor targeting VAMP1 illustrate that point.

To be a good *in vitro* alternative to the mouse lethality bioassay (MLB), assay sensitivity is a crucial parameter. It has been reported that the MLB has a LD50 range of 5-10 pg/ml for botulinum toxin [30] and here, we reached an EC50 of 40 fg/ml (=272 fM) for BoNT/A. While the cell-based assay developed here has thus the necessary sensitivity to serve a batch-release cell-based assay, further validation will be required to use it for such purposes.

The sensitivities measured here for BoNT/A and E are similar as those obtained by western blot cleavage assay in the same cellular model after 14 days of culture [19]. Interestingly, in both studies, BoNT/E is more potent compare to A. This does not fit with previous experiments done on primary rodent cells [31] that indicated that BoNT/A was 30 times more potent than BoNT/E. But our findings match with the observations from the first clinical trial in human (phase one study) in which it was shown that BoNT/E potency is at least equivalent or even greater compare to toxin A [24]. These observations underline the translational value of a human cellular model.

Finally, to prove we can use our system to test different steps of toxin intoxication, we tried to modulate toxin binding by a pre-exposure of our cells with gangliosides. As it was previously shown for iCell GABA neurons [14], we found that GT1b addition also improves BoNT/A potency in MNs (Figure4A). This

observation differs from earlier findings which indicated that pre-exposure with gangliosides on iCell MNs did not impact their sensitivity to BoNT/A [17]. This discrepancy could be explained by the difference in nature of BoNT, protocols for gangliosides and toxin treatment. In the previous study, iCell MNs were pre-treated with 45µM GT1b for 24 hours prior to natural BoNT/A (produced by Clostridium) exposure while in our study, cells were pre-treated with 30 µM for 48h prior to recombinant BoNT/A exposure. Apart from these small differences, another important parameter to differentiate these two experiments is the readout which is employed to quantify the action of BoNTs. This suggests that luminescent tool is more sensitive to small changes in BoNT cleavage compare to Western blot.

We also showed that our assay can be used to study toxin translocation. The blockage of this cellular process in our cells, completely inhibited rBoNT/A activity.

Nevertheless, the CB assay presented here can be improved. One way to do it is to generate a stable hiPSC line expressing the SNAP25 luminescent probe. From these stem cells, it would be possible to generate specialized neurons (MNs, glutamatergic neurons, sensory neurons...) bankable as precursors. This approach would overcome the usage of lentivirus, limit number of manipulations and reduce time of experiment. All these improvements will facilitate the use of these engineered cells in a QC environment and/or for high-throughput screening.

Abbreviations

BoNT, Botulinum neurotoxin, SNAP-25, Synaptosomal-associated protein of 25 kDa, hiPSC, human induced Pluripotent Stem Cells, SNARE, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor, SV2, synaptic vesicle proteins, MLB, mouse lethality bioassay, LD₅₀, toxin's dose lethal for half of the animal injected, CB-assay, cell-based assays, FRET, Förster resonance energy transfer, Concanamycin A, EC₅₀, Half maximal effective concentration, MNs, motor neurons.

Declarations

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Conflict of interest : Harvard Medical School has filed a patent application covering the split nanoluc-based assays for detecting botulinum neurotoxins, with F.Y. and M.D. as co-inventors.

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Figures

Figure 1

Split luciferase sensors for detecting BoNT activity. (A) Schematic representation of the split luciferase-based biosensor. The split NANOLUC™ luciferase is linked together by a linker containing a SNARE sequence. By cleaving the SNARE part of the sensor, BoNTs separate NANOLUC™ luciferase and prevent it from reconstitution into a functional luciferase. The decrease of luminescence is inversely proportional to the toxin activity. (B) Schematic drawing of three sensor proteins designed for BoNTs detection. For SNAP25-cleaving BoNT, one is NNano-SV2C-p25-CNano whereas the other one is NNano-p25-CNano. For VAMP1-cleaving BoNT, the sensor protein Nnano-vp1-Cnano was prepared. (C) In vitro detection assay of BoNT/A. Sensor proteins (30 nM) were mixed with different concentrations of BoNT/A. The control sample is no toxin added. The curve was plotted based on the percentage of luminescence of Nanoluc luciferase by using toxin added sample/ control sample. Negative control was also performed mixing BoNT/A with Nnano-vp1-Cnano sensor protein. Assay was preformed duplicate (D) In vitro detection assay of BoTN/B. The process was the same as described for detecting BoNT/A. Negative control was also performed mixing BoNT/B with Nnano-SV2C-p25-Cnano sensor protein. Assay was preformed duplicate.

Figure 2

Split NANOLUC™ luciferase sensor can be used in rodent and human neurons(A) Schematic drawing of lentiviral vector containing two hSYN promoter/WRPE cassettes for constitutive expression of BoNT sensor4 and GFP in neurons. (B) Detection assay of BoNT/A using virus transduced rodent neuronal cells. The remaining luciferase activity was measured. Assay was preformed triplicate (C) Comparison of iCell MN toxin sensitivity to BoNT/A at day 7 and day 14-15. Graph shows dose response curve of SNAP25 cleavage. Data points are mean of n = 3 independent experiments performed in triplicate. (D-E) Impact of lentiviral transduction (MOI 16) on MN identity 6 d after transfection. ISLET1-positive MNs were counted from an average of 2000-4000 cells in random fields from 3 independent experiments. (D) GFP (green); Immunostaining of DAPI (blue), Islet1(purple) in iCellMN. Scale bar 100 μ m. (E) Number of Islet positive cells normalized by the number of cells. Statistical two-tailed Student's t-tests: *P 0.05; **P 0.01; ***P 0.001; NS, P>0.05 not significant (NS). Error bars show SEM.

Figure 3

Cell-based assay using the split NANOLUC™ luciferase sensor. (A) Protocol of the CB-assay using the BoNT sensor1. (B-C) Comparison of rBoNT/A (0), rBoNT/A and rBoNT/E potency in the BoNTsensor4 CB-assay. Data points are mean of n=4 independent experiments in triplicate. (B) Graph shows concentration response curves. (C) Comparison of the pEC50 of rBoNT/A and E calculated from curves. Statistical two-tailed Student's t-tests: *P 0.05; **P 0.01; ***P 0.001; NS, P>0.05 not significant (NS). Error bars show SEM.

Figure 4

Mechanistic assay using the CB assay.(A) Increasing cell uptake by GT1b addition. rBoNT/A (1pM) was incubated 24h in absence or presence of GT1b and the action of the toxin was determined by measuring the percentage of BoNT sensor4 cleavage compare to the basal conditions. Data points are mean of n=4 independent experiments in triplicate. (B) Blocking of toxin action by Con A. rBoNT/A (1nM) was incubated 10 minutes in stimulating conditions (60nM KCl). After toxin wash out and 4h in absence or presence of ConA, the percentage of BoNT sensor4 cleavage was compared to the basal conditions. Data points are mean of n=4 independent experiments in triplicate. Statistical ordinary one-way ANOVA multiple comparisons: *P 0.05; **P 0.01; ***P 0.001; ****P 0.0001; NS, P>0.05 not significant (NS). Error bars show SEM.

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