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Assessment of ELISA as Endpoint in Neuronal Cell-based Assay for BoNT Detection using hiPSC derived neurons

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Abstract

Introduction—Botulinum neurotoxins (BoNTs), the causative agents of botulism, are widely used as powerful bio-pharmaceuticals to treat neuro-muscular disorders. Due to the high potency and potential lethality of BoNTs, careful monitoring of the biologic activity of BoNT-based pharmaceuticals is required to ensure safe usage. For decades, the only approved method for potency determination of pharmaceutical BoNTs was the mouse bioassay (MBA), but in recent years improvements in cell-assay technologies have enabled MBA replacement by cell-based assays for specific product evaluations. This project details a method for quantitative and sensitive detection of biologic activity of BoNT/A1 in human induced pluripotent stem cell (hiPSC) derived neurons using an ELISA as a method to determine SNAP-25 cleavage by BoNT/A1 following toxin exposure.

Methods—HiPSC derived neurons from two different sources were exposed to serial dilutions of BoNT/A1, and quantitative detection of toxin activity was evaluated and optimized in cell lysates using ELISA to detect cleaved SNAP-25.

Results—The results from this study indicate that an ELISA using ultra TMB as a substrate quantitatively detects cleaved SNAP-25 in cell lysates of BoNT/A1 exposed hiPSC-derived neuronal cells with similar or greater sensitivity as Western blot (EC₅₀ ~ 0.3 U / well).

Discussion—This study demonstrates a human specific and sensitive cell-based detection platform of BoNT/A1 activity using ELISA as an endpoint for quantitative detection of the SNAP-25 cleavage product. This assay is applicable to moderate to high-throughput formats and importantly employs non-cancerous human-specific neuronal cells for potency evaluation of a bio-pharmaceutical for human use.

Keywords

botulinum neurotoxin; cell based assay; detection; ELISA; hiPSC derived neurons; stem cells

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Introduction

Botulinum neurotoxins (BoNTs) are the causative agent of botulism, but are also widely employed as powerful pharmaceutical agents to treat a variety of neuromuscular disorders as well as other neuronal syndromes such as hyperhidrosis, chronic migraines, neurogenic bladder, and more. (Dressler, 2012). Traditionally, the potencies of pharmacological preparations of BoNTs have been assessed by the *in vivo* mouse bioassay to determine toxin activity for product stability, lot to lot consistency, and product release (Hatheway, 1988; Schantz and Kautter, 1978). However, due to ethical and biosafety concerns regarding the use of large numbers of mice, the Food and Drug Administration (FDA) and manufacturers of pharmaceutical BoNTs have been putting forth efforts to replace the mouse bioassay (Adler, et al., 2010; Sesardic, 2007). Neuronal cell-based assays (NCB assays) have emerged as a feasible alternative in recent years, due to the sensitive and specific detection of holotoxin activity in several neuronal cell models and the ability to standardize these assays (Fernandez-Salas, et al., 2012; Hubbard, et al., 2012; Kiris, et al., 2014; Pellett, 2013; Whitmarsh, et al., 2012a). Unlike assays that determine individual steps in the BoNT intoxication process, NCB assays determine all four steps which is essential for API final characterization. The largest manufacturer of pharmaceutical BoNTs in the USA, Allergan developed a cell based assay for product release in 2012 using the human neuroblastoma cell line SIMA cells (Fernandez-Salas, et al., 2012). Other efforts have utilized human induced pluripotent stem cell (hiPSC) derived neurons for sensitive detection of BoNT activity in a human non-cancerous cell model (Whitmarsh, et al., 2012).

A useful method to determine the endpoint for a cell-based assay for BoNT/A activity is Western blot detecting cleaved and uncleaved SNAP-25 (synaptosomal associated protein 25). While this endpoint is appropriate and useful for research purposes, the semi-quantitative nature of Western blots and the laborious and costly steps involved make this end-point less well suited for a product release assay. Alternatively, quantitative and scalable ELISA platforms for detecting intracellularly cleaved SNAP-25 in cell lysates have been developed (Fernandez-Salas, et al., 2012; Nuss, et al., 2010). However, ELISA platforms are dependent on the availability of an antibody that detects only BoNT/A cleaved SNAP-25, and an optimized protocol for the cell model used. In a recent report, an NCB-assay combined with an ELISA endpoint for detection of cleaved SNAP-25 has been described using mouse embryonic stem cell derived neurons (Yadirgi et al., 2013). However, the same method was not directly transferrable to hiPSC derived neurons due to low detection levels. Here a modification of the method is described that allows the sensitive detection of BoNT/A holotoxin activity in hiPSC derived neurons using quantitative ELISA based detection of cleaved SNAP-25 as an endpoint. Assessment of the NCB assay-ELISA method in two different hiPSC derived cell models showed that this method is as or more sensitive in detecting BoNT/A holotoxin activity as an NCB assay using Western blot to detect the cleaved SNAP-25.

Materials and Methods

Botulinum neurotoxin

Isolated pure 150 kDa BoNT/A1 toxin heterodimer was isolated from the *Clostridium botulinum* strain Hall A-hyper essentially as previously described (Malizio, Goodnough, & Johnson, 2000). Specific activity was determined by mouse bioassay (MBA) (Hatheway, 1988; Schantz & Kautter, 1978) to be 1.25×10^8 mouse LD₅₀S (Units)/mg.

Reagents and antibodies

For the ELISA, the Polystyrene Maxisorp plates (Nunc) or Immulon Immunoassay plates (Thermo Scientific) were used as indicated. The antibodies were all provided by the Sesardic lab. The BoNT/A cleavage site-specific anti-peptide antibody against SNAP25 190–197 was as previously described (Ekong, Feavers, & Sesardic, 1997) and was affinity purified. This antibody has previously been shown to bind only BoNT/A cleaved SNAP-25 in both the ELISA and Western blot assays (Ekong, Feavers, & Sesardic, 1997), Jones et al., 2008). Anti SNAP25 detecting and signal amplification antibodies were made in sheep against SNAP25 1–57 and SNAP25 111–157, respectively (Liu et al., 2012). Rabbit anti-sheep HRP was from Thermo Scientific. All SDS-PAGE gel reagents and Western blot reagents were purchased from Life Technologies, and other chemicals and reagents were purchased from SIGMA unless otherwise noted. The BCA assay was purchased from Pierce (Thermo Fisher) and used according to manufacturer's instructions.

Neuronal cells

Human induced pluripotent stem cell (hiPSC)-derived neurons (iCell Neurons, Cellular Dynamics International, Madison, WI) were prepared and maintained in the manufacturer's provided serum-free medium as previously described (Pellett, Tepp, Clancy, Borodic, & Johnson, 2007; Pellett, Tepp, Toth, & Johnson, 2010; Whitmarsh, et al., 2012a). Cells were seeded into 0.01% poly-L-ornithine (PLO) (SIGMA) and 8.3 µg/cm² matrigel (BD Biosciences) coated 96-well Techno Plastic Products (TPP) plates at a density of ~35,000 cells/well. The cells were used in the toxin assay at 5 days post plating. HIP Neurons and accompanying media were supplied from Global Stem (Rockville, MD) and grown according to manufacturer's instructions with the exception that they were plated onto PLO-laminin-coated plates before differentiation, and the differentiation medium was supplemented with 2 µM all-trans retinoic acid (Stemgent, Cambridge, MA) and 5 µM purmorphamine (CalBiochem). After 5 days of differentiation, the cells were kept in the same plate and differentiation media was replaced with maintenance media (supplied by Globalstem). The neurons were matured for 18 days prior to use in the toxin assays. Primary mouse spinal cord cells were prepared as previously described (Pellett, et al., 2015; Whitmarsh, et al., 2013) and cultured for 18 days prior to use in a toxin assay. All cell types were incubated at 37°C in a humidified 5% CO₂ atmosphere. To produce bulk cell lysates containing cleaved SNAP-25 or only uncleaved SNAP-25 for use in ELISA optimization, the primary mouse spinal cord cells were either exposed to 10 U BoNT/A1 per well or no toxin in 50 µl culture media for 48 h. After 48 h, cells from 48 wells, respectively, were lysed for ELISA as described and the cell lysates were pooled. An aliquot of the lysates was

examined by Western blot to confirm over 90 % cleaved SNAP-25 in toxin exposed and uncleaved SNAP-25 in unexposed cell lysates.

For the toxin exposure, serial dilutions of BoNT/A1 were added to the cells as indicated, in a volume of 50 µl / well, and cells were incubated for 48 h before lysis for the ELISA or Western blot. Cell lysates were prepared from the BoNT/A1 exposed cells by aspirating the toxin containing media and washing the cells once with 75 µl of 10 mM Hepes buffer pre-warmed to 37°C. Hepes was removed and 105 µl of room temperature M-PER (Mammalian protein extraction reagent) lysis buffer (Pierce) containing 154 mM NaCl was added. The plates were shaken on a plate shaker for 10 min. at 700 rpm, and 80 µl per sample were immediately frozen at -70°C for the ELISA. The remainder of the sample was mixed with 8.3 µl of 4 x LDS sample buffer (Life Technologies) for Western blot analysis.

ELISA

The frozen samples were thawed at room temperature immediately before the ELISA, and any remaining cell debris was removed by centrifugation prior to conducting the assay. The ELISA was performed as described below, except where noted otherwise. To increase sensitivity, TMB ultra substrate (Pierce) was used instead of ABTS where indicated.

Ninety six-well ELISA plates (Nunc, Maxisorb) were coated with 50 µl/well of 5 µg/ml affinity purified capture antibody against SNAP25 190–197 in carbonate buffer (50 mM sodium carbonate, pH 9.6), overnight at +4°C. After carefully removing buffer by decanting, the plates were blocked with 250 µl per well of 2.5% (w/v) skimmed milk powder (Marvel) in PBST (phosphate buffered saline with 0.05% v/v Tween-20) (M-PBST or blocking buffer) for 90 min at room temperature (RT). The plates were washed at least three times with PBST using a plate washer. Cell lysate (50 µl) was added to the plates, together with 50 µl of M-PBST, and placed on a shaker at 400 rpm for 10 min followed by resting for 90 min at RT. M-PBST (100 µl per well) was added to blank controls (negative control). In all experiments, cell lysate from cells not exposed to toxin was added as a second negative control. Internal controls for the ELISA included 'no capture antibody' and 'no detection antibody' (primary and secondary) were also included during the assay optimization. Plates were washed three times with PBST and detection of captured truncated SNAP-25 was performed by addition of an equal mixture of sheep anti-SNAP25 antibodies directed to SNAP25 1–57 and 111–157, respectively, in M-PBST at 100 µl per well. After incubation for 90 min at RT, the plates were again washed three times with PBST and the reaction visualized with 100 µl per well of commercially sourced rabbit anti-sheep HRP conjugated antibody (Thermo Scientific) in M-PBST and incubation for 90 min at RT. After washing with PBST 200 µl/well of substrate solution (50 mM citric acid pH4.0, 0.05% w/v ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, and 0.05% v/v of a 30% w/v Hydrogen peroxide solution) was added to all wells and the colour allowed to develop at RT for 30 min. Following colour development, the plates were briefly shaken and absorbance determined at 405 nm using a plate reader (Multiscan plate reader). The optimized protocol is shown in box 1.

Box 1

Optimized ELISA protocol for hiPSC derived neurons after toxin exposure of cells:

- Expose hiPSC derived neurons to serial dilutions of BoNT/A for 48 h. Always add a no-toxin control.
- Gently aspirate media + toxin
- Wash cells once with 75 μ l 37°C Hepes (10 mM, pH 7.2)
- Add 105 μ l of M-PER lysis buffer + 154 mM NaCl at RT
- Shake on plate shaker for 10 min at 700 rpm. Inspect to ensure full lysis and shake longer if necessary.
- Transfer 80 μ l per sample to 96-well tubes, and immediately freeze at -70°C (ELISA samples)
- Optional: Transfer 20 μ l to strip tubes containing 7 μ l of 4 x LDS sample buffer (Western samples)
- Coat 96-well microtiter plate (Polystyrene Maxisorp) using 50 μ l per well of the rabbit affinity purified anti-SNAP25 (aa 190–197) at 5 $\mu\text{g}/\text{ml}$ in carbonate buffer (50 mM sodium carbonate, pH 9.6). Shake briefly.
- Cover plate using transparent ‘adhesive film’ and incubate overnight at 4°C .
- Empty wells and add 300 μ l blocking solution (2.5 % w/v Skimmed milk powder, PBS, 0.05% Tween 20) seal plate and incubate for 1 hour at RT.
- Wash plate three times with PBS Tween (phosphate buffered saline, 0.05 % Tween 20).
- Add 50 μ l of blocking solution to all wells.
- Centrifuge cell lysates for 1 min at max speed in a microfuge to remove any cellular debris, and add a 50 μ l cell lysate per well, cover with adhesive film. Always include the no-toxin controls (cell lysates of non-exposed cells) and a ‘no-lysate’ control (add 50 μ l M-PER lysis buffer + 154 mM NaCl instead of cell lysates).
- Place on plate shaker at 400 rpm for 10 min followed by incubation for 90 min at RT.
- Wash plate 5 times with PBS Tween.
- Add 100 μ l of sheep anti-SNAP25 (raised to SNAP25 epitopes 1–57, 111–157) at a 1:1,000 dilution to each well (e.g. 15 μ l of stock mix plus 15 ml of blocking solution).
- Incubate for 90 min at RT. Wash 5 times.

- Add 100 µl rabbit anti-sheep HRP conjugated antibody (Thermo, Code No. 31480) at a 1:16,000 dilution (e.g. 2 µl of stock in 32 ml of blocking solution).
- Incubate for 90 min at RT. Wash 5 times.
- Add 100 µl of TMB Ultra substrate (Thermo Scientific Pierce) and incubate for 10–15 minutes at RT.
- Add 100 µl of 2 M sulphuric acid to stop reaction, and measure absorbance at 450 nm.
- Subtract the background value from the ‘no lysate’ control. The ‘no-toxin’ control should be zero.

Western blot analysis

For Western blot analysis of cell lysates, 4X lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) was added to the same cell lysates that were used in the ELISA, and the samples were analyzed by Western immunoblots for SNAP-25 cleavage as previously described (Pellett, et al., 2007; Pellett, et al., 2010; Whitemarsh, et al., 2012a). Cleaved and uncleaved SNAP-25 bands were quantified by densitometry using a Foto/Analyst FX system and TotalLab Quant software (Fotodyne). Data plots and EC₅₀ values (four parameters – variable slope) were generated using PRISM 6 software.

Results

The cleaved SNAP-25 ELISA is concentration dependent, and the signal can be increased dramatically by using ultra TMB as substrate

In order to examine whether a previously developed ELISA method (R.G.A. Jones, personal communication) can be used to detect intracellularly cleaved SNAP-25 in a sensitive hiPSC derived neurons cell model (Pellett, et al., 2015; Whitemarsh, et al., 2012), detection of cleaved SNAP-25 in cell lysates from hiPSC derived neurons exposed to serial dilutions of BoNT/A1 was compared by ELISA and Western blot. While the Western blot data indicated dose-dependent SNAP-25 cleavage ranging from 100 % to 0 % cleavage as previously described (Whitemarsh, et al., 2012), the ELISA resulted in a very low and non-quantifiable signal (data not shown).

In order to further assess the ELISA and attempt to improve sensitivity, the concentration dependence of the ELISA was examined using bulk cell lysates prepared from primary mouse spinal cord cells that either contained mostly cleaved SNAP-25 or exclusively uncleaved SNAP-25. Total protein concentration in the cell lysates were determined by BCA assay according to manufacturer instructions. Serial dilutions of each cell lysate in the range of 0.15 to 10 µg / well (about equivalent to the total protein amount that can be loaded for hiPSC derived neurons) were prepared in lysis buffer and analysed by the same ELISA protocol. A concentration dependent dose-response was observed, however, the maximum signal was too low to yield a quantifiable measure. Untreated cell lysates did not result in any significant signal, indicating a low background signal.

To determine whether the ELISA signal can be increased to allow for more sensitive detection of cleaved SNAP-25, two parameters were tested. First, the cell lysates were incubated with the capture antibody coated plate overnight, and second, a more sensitive HRP substrate, ultra TMB, was employed. While the overnight incubation increased sensitivity only slightly, it also increased the background signal of the samples containing cell lysates not treated with BoNT/A1 (data not shown). Not surprisingly, changing the HRP substrate from ABST to ultra TMB increased sensitivity dramatically, and resulted in a long linear dose-response relationship from 0.3 to 10 μg total cell lysate per well (Figure 1), indicating that by using a sensitive ELISA substrate this assay can be used even on samples with relatively low protein concentrations.

The modified ELISA quantitatively detects cleaved SNAP-25 in human iPSC-derived neurons cell lysates

The modified ELISA protocol (using ultra TMB as substrate) was assessed for detection of cleaved SNAP-25 in hiPSC derived neuron (HIP Neuron) lysates. Equal amounts of total protein per well (12 μg , as assessed by BCA assay) of lysates from differentiated neurons exposed to serial dilutions of BoNT/A1 resulted in a good dose-response curve, however, the error was large (not shown). To reduce the error associated with this assay, the following factors were assessed: (1) loading of the same volume of cell lysate rather than adjusting for same total protein concentration was evaluated since the same number of cells are seeded into the tissue culture plates and the total protein concentration in the cell lysates was very similar $285 \pm 36.7 \mu\text{g} / \text{ml}$ (average and standard deviation); (2) the outer rows on the ELISA plates were left empty to avoid 'edge effects'; (3) two different ELISA plates, Polystyrene Maxisorp plate (Nunc) or an Immulon Immunoassay plate (Thermo Scientific), were assessed. In a direct comparison of the ELISA using 50 μl cell lysates per well, no outermost rows, and either a Polystyrene Maxisorp plate (Nunc) or an Immulon Immunoassay plate (Thermo Scientific) in parallel, both plates yielded good dose-response curves but the standard deviations on the Immulon plate were smaller for all samples (Fig 2). This indicates that this ELISA format can be used to quantitatively detect cleaved SNAP-25 in cell lysates from hiPSC derived neurons when ultra-TMB was used as a substrate, Immulon Immunoassay plates are used, the same volume of cell lysates is loaded, and the outer rows of the ELISA plate are omitted.

The optimized ELISA detects cleaved SNAP-25 in human iPSC-derived neurons cell lysates with similar or greater sensitivity as Western blot, and sensitivity increases with increasing SNAP-25 expression

The optimized ELISA protocol was used to compare detection of BoNT/A1 cleaved SNAP-25 in iCell Neurons and HIP Neurons after exposure to serial dilutions of BoNT/A1 of each with detection by Western blot and densitometry. While previous data has indicated greater sensitivity of iCell Neurons compared to HIP Neurons (Pellett, et al., 2015; Whitmarsh, et al., 2013), the HIP Neurons can be seeded at greater density resulting in higher total protein concentration in cell lysates. The protein concentrations of the cell lysates were determined to be 90.4 ± 12.2 and $285 \pm 36.7 \mu\text{g} / \text{ml}$ for iCell Neurons and HIP Neurons, respectively. Both assay end-points resulted in sigmoidal dose-response curves for both cell types with relatively small standard deviations. The ELISA EC_{50} values for both

cell types was around ~ 0.34 U BoNT/A1 / well, while the Western blot EC₅₀ value was about 3-fold greater for HIP Neurons (~1.5 U BoNT/A1 / well for HIP Neurons versus ~0.49 U BoNT/A1 / well for iCell Neurons) (Figure 3). The 95 % confidence interval was slightly wider for the ELISA than for the Western blot (Figure 3). These data indicates that the ELISA detects cleaved SNAP-25 with similar (iCell Neurons) or greater (HIP Neurons) sensitivity than the Western blot, and that the ELISA endpoint detects cleaved SNAP-25 in iCell Neurons and HIP Neurons with equal sensitivity, while the Western blot is the more sensitive in iCell Neurons than in HIP Neurons.

Discussion

Cell-based BoNT/A detection methods have gained in importance in recent years for both research purposes and for potency determination of pharmaceutical BoNT/A1 preparations, (Fernandez-Salas, et al., 2012; Kiris, et al., 2014; Pellett, 2013; Whitmarsh, et al., 2012a). Several endpoints for detection of BoNT activity can be used in cell-based assays, including determination of the percentage of SNARE cleavage by Western blot, measurements of neurotransmitter or FM dye release, electrophysiological measurements, or detection of SNARE cleavage products by ELISA. Detection of SNARE cleavage or cleavage products is specific and quantitative and therefore is a desirable endpoint for potency determination of pharmaceutical BoNTs. Western blots to determine the ratio of uncleaved to cleaved SNAP-25 in primary neurons or hiPSC derived neurons has been shown to yield a sensitive, quantitative, and reproducible platform for BoNT detection (Pellett, 2013; Pellett, et al., 2010; Whitmarsh, et al., 2012). However, the labour intensive and costly Western blots do not lend themselves to high throughput screening. Therefore, ELISA based detection methods for the SNAP-25 cleavage product have been developed for quantitation of BoNT/A1 in various cell models (Fernandez-Salas, et al., 2012; Nuss, et al., 2010). However, this ELISA detection platform has not been developed for the highly sensitive and human specific hiPSC derived neuron cell models (Pellett, et al., 2015; Whitmarsh, et al., 2012a). Initial analyses of an ELISA based detection system in hiPSC derived neurons (iCell Neurons, Cellular Dynamics Inc.) and HIP Neurons (Globalstem) resulted in a low and non-quantifiable signal. The same ELISA protocol had previously yielded a quantifiable measure of cleaved SNAP-25 in other biochemical models as well as in mouse embryonic stem cell derived neurons (Liu, et al., 2012; Yadirgi et al., 2013). The goal of this study was to modify this existing ELISA protocol for use in hiPSC derived neurons.

Direct protocol modifications such as use of a more sensitive substrate and selection of the ELISA plate resulted in quantitative detection of BoNT/A1 cleaved SNAP-25 in hiPSC derived cell lysates (Fig. 1–3, Box 1). Direct comparison of the ELISA detection method and detection by Western blot indicated that the ELISA method is as or more sensitive than the Western blot / densitometry method to quantitatively detect BoNT/A1 cleaved SNAP-25 in two human induced pluripotent stem cell derived neurons, iCell Neurons (Cellular Dynamics Inc) and differentiated HIP Neurons (Globalstem). Further, the data indicate that unlike the Western blot, which detects SNAP-25 cleavage with an about 5-fold greater sensitivity in iCell Neurons compared to HIP Neurons (Whitmarsh, et al., 2013), sensitivity of the ELISA detection method is the same for both cell models, with an EC₅₀ of about 0.3 U / well (Figure 3). This may be due to the three times greater protein expression levels in

HIP Neurons, since the ELISA detects cleaved SNAP-25 only whereas the Western blot method determines the ratio of cleaved versus uncleaved SNAP-25. In addition, differences in antibody sensitivity and signal amplification by the dual detection system in the ELISA may contribute to greater sensitivity of the ELISA method in the HIP neuronal assay. The error using the ELISA method is similar to that using the Western blot/densitometry method. However, several critical factors need to be considered when using the ELISA as an end-point: (1) The same amount of cells needs to be seeded into each well to ensure consistent protein concentration in cell lysates; (2) Care must be taken to not wash out cells during the assay, as the signal is concentration dependent ; (3) The ELISA plate and substrate should be carefully selected based on the cell type used and total protein concentration of cell lysates; (4) a BCA assay or a second ELISA for a control protein should be included as a control step to ensure loading of equal protein concentration. Samples that fall outside of a pre-determined parameter should then be removed from the quantification, thereby eliminating errors due to unequal loading.

In summary, this study demonstrates a human specific and sensitive cell-based detection platform of BoNT/A1 activity using ELISA as an endpoint for quantitative detection of the SNAP-25 cleavage product.

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Figure 1.

Substrate selection for ELISA. A: Primary mouse spinal cord cells were exposed to 10 U / 50 µl / well of BoNT/A1 for 48 h, and serial dilutions of cell lysates were analyzed for cleaved SNAP-25 by ELISA using ABTS or ultra TMB as substrate. Absorbance values at A405 are shown for the ABTS substrate and at A450 for the ultra TMB substrate. The graph was created as an XY scatter blot in Prism 6.

Figure 2.

Plate selection for ELISA. HIP Neurons were exposed to serial dilutions of BoNT/A1, and cell lysates were analyzed for cleaved SNAP-25 by ELISA using either a Nunc Polystyrene Maxisorp plate (Nunc) or a Thermo Scientific Immulon Immunoassay plate (Immunlon). The signal was detected with ultra TMB. The average and standard deviations of 4 replicates is shown. Dose response curves were fitted using Prims 6 software and a nonlinear regression (variable slope, four parameter).

Figure 3.

Comparison of ELISA and Western blot endpoints of the CBA. HIP Neurons or iCell Neurons were exposed to serial dilutions of BoNT/A1, and cell lysates were analyzed for cleaved SNAP-25 by ELISA using the optimized protocol or by Western blot. The average and standard deviations of 6 replicates are shown, respectively. Dose response curves were fitted using Prims 6 software and a nonlinear regression (variable slope, four parameter). EC₅₀ values and the 95 % confidence intervals for the EC₅₀ values were computed from the regression analysis and are shown below the graph. A representative Western blot image is shown in (B).