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Sensitive and quantitative detection of botulinum neurotoxin in neurons derived from mouse embryonic stem cells

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ABSTRACT

Botulinum neurotoxins (BoNTs), the most poisonous protein toxins known, represent a serious bioterrorism threat but are also used as a unique and important bio-pharmaceutical to treat an increasing myriad of neurological disorders. The only currently accepted detection method by the United States Food and Drug Administration for biological activity of BoNTs and for potency determination of pharmaceutical preparations is the mouse bioassay (MBA). Recent advances have indicated that cell-based assays using primary neuronal cells can provide an equally sensitive and robust detection platform as the MBA to reliably and quantitatively detect biologically active BoNTs. This study reports for the first time a BoNT detection assay using mouse embryonic stem cells to produce a neuronal cell culture. The data presented indicate that this assay can reliably detect BoNT/A with a similar sensitivity as the MBA.

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1. Introduction

Botulinum neurotoxins (BoNTs) are the most poisonous naturally occurring protein toxins known to humankind, and are the causative agent of the human disease botulism. BoNTs are primarily produced by bacteria of the genus *Clostridium botulinum* [1,2]. While botulism cases are rare in the USA [3], the extraordinary potency and biological properties of BoNTs have lead to marked significance of BoNTs as both a potential bioterrorism weapon [4] as well as an extremely useful pharmaceutical in treatment of neuromuscular disorders and cosmetics [5,6].

BoNTs are comprised of a large group of proteins that are subdivided into 7 different serotypes (A–G) based on their structure and biological properties, with most serotypes being further subdivided into several subtypes [7,8]. The 150 kDa proteins consist of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) that are linked by a disulfide bond [9]. The toxins specifically enter motor neurons by binding to protein and ganglioside cell surface receptors via the C-terminal receptor binding domain of the HC (H_C), which induces endocytosis of the toxins into endosomes [10,11]. Inside the vesicle, protonation causes a conformational change of the HC and insertion into the endosomal membrane via the N-terminal translocation domain of the HC (H_N), leading to the formation of a trans-membrane protein conducting channel and translocation of the LC through the HC channel into the cytosol [12,13]. The LC, which is a zinc-endopeptidase, then specifically cleaves and thereby inactivates soluble *N*-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins inside the cell's cytosol [14,15]. SNARE proteins are essential for neurotransmitter release by catalyzing membrane fusion [16], and cleavage of a SNARE protein leads to a block of neurotransmitter release and inactivation of the affected neuromuscular junction and ultimately results in paralysis of the affected muscle. Of the seven serotypes of BoNTs, BoNT/A and E cleaving SNAP-25 at distinct sites [17,18], BoNT/B, D, F, and G cleave synaptobrevin (also known as VAMP) and BoNT/C cleaves both syntaxin and SNAP-25 [14,15,19–21].

The presence and potency of biologically active botulinum neurotoxin is currently evaluated by the mouse bioassay [22,23], in which mice are injected with the testing substance and observed for signs of BoNT specific toxicity and death. Because of the large number of animals required for this assay, and other disadvantages such as a large error, lab to lab variations, high cost, the requirement of 4 days for completion of the assay, an alternative assay would be of tremendous value. While many *in vitro* assays for detection of BoNTs or BoNT catalytic activity have been developed (reviewed in [24,25]), the only alternative assay that requires all biological functions of BoNTs and is equally sensitive as the mouse bioassay is a cell-based assay using primary rat spinal cord cells [26–28]. This assay correlates well with the mouse bioassay, but is slightly more sensitive and has a lower error than the MBA; however, it requires the preparation of primary cells for each

Abbreviations: BoNT, botulinum neurotoxin; MBA, mouse bioassay.

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experiment and would not be convenient for repeated testing [27]. Here we report for the first time the use of mouse embryonic stem cells differentiated to neurons as an alternative to primary cells. Our data indicate that these cells provide sensitivity similar to the MBA, and that cell preparation and BoNT activity assays can be standardized to yield reproducible results.

2. Materials and methods

2.1. Reagents and media

All tissue culture media, solutions, and media components, as well as growth factors, were purchased from Invitrogen (Carlsbad, CA). Mouse laminin and purmorphamine were purchased from Stemgent (San Diego, CA). Retinoic acid, beta-mercaptoethanol, and leukemia inhibitory factor (LIF) were purchased from Sigma–Aldrich (St. Louis, MO). Cyclic AMP (cAMP) was purchased from Promega (Madison, WI). Acutase/0.5 mM EDTA was purchased from Innovative Cell Technologies (ICT) (San Diego, CA).

2.2. Mouse embryonic stem cell culture

The mouse embryonic stem (mES) cell line Mnx1-RFP was established by transfecting a vector driving red fluorescent protein (RFP) expression under the 3.5 kb Mnx1 (also known as HB9) gene promoter [29]. The cells were maintained and differentiated essentially as described previously in a 37 °C tissue culture incubator at 5% CO_2 [30–33]. In short, cells were maintained in MES medium [DMEM/F12, 15% fetal bovine serum, 1× non-essential amino acids, sodium pyruvate (1 mM), 1× GlutaMAX, 2-mercaptoethanol (0.1 mM) and human recombinant leukemia inhibitory factor (LIF, 1000 U/ml)] (all reagents from Gibco, Rockville, MD) on TPP 10 cm^2 tiny flasks (Midsci, St. Louis, MO) that had been coated for 1 h at 37 °C 0.1% gelatin. Medium was replaced every 2 days, then cells were split by trypsin–EDTA digestion, and $\sim 5 \times 10^4$ cells were plated into a fresh gelatin coated flask. It was essential for successful differentiation that the cells were not over-grown at this stage. For differentiation to neurons, $\sim 2–4 \times 10^6$ cells were transferred to a 100 mm Petri dish (Fisher Scientific) in 15 ml of MES/SR (same as MES, but with 15% knockout serum replacement instead of FBS, and no LIF) in order to induce embryoid body formation. After 2 days, the MES/SR was replaced with MES/SR containing 0.5 μM retinoic acid and 1 μM purmorphamine (unless otherwise noted in the text). The medium was renewed after 3 days, and after an additional day of incubation the embryoid bodies were digested with acutase/0.5 mM EDTA, and 75,000 cells/well were plated into 96-well TPP dishes (Midsci, St. Louis, MO) coated with 1 μg/well of laminin in NS medium (Neurobasal supplemented with 1× B27, 1 μM cAMP and 20 ng/ml GDNF) to maintain neurons. The cells were maintained in culture for 9 days before use in toxin assays. For microscopy analysis of EBs, the EBs were washed three times in PBS, and placed on a microscope slide. To analyze the plated cells by microscopy, the cells were plated onto laminin coated glass cover-slips. The cells were maintained in culture for 9 days before use in toxin assays.

2.3. Botulinum neurotoxin

Pure botulinum neurotoxin (BoNT) A, and E (150 kDa) were prepared from *C. botulinum* strains Hall A hyper, and Alaska E (E43) as previously described [34,35]. The toxins were dissolved in phosphate buffered saline, pH 7.4 and 40% glycerol, and stored at −20 °C until use. Activity of the BoNT/A, and E preparations were determined by the mouse bioassay [22,23], and specific toxicity was about 1.3×10^8 mouse LD_{50} U/mg for BoNT/A, and 0.76×10^8 mouse LD_{50} U/mg for BoNT/E.

2.4. BoNT activity assay

The biologic activity of BoNT was assessed in the cultured neurons by exposing the cells to the indicated amounts of purified BoNT/A in NS medium without GDNF for the indicated times at 37 °C, 5% CO_2. Cells were harvested by lysis in 75 μl of 1× LDS sample buffer (Invitrogen), and cell lysates were analyzed for SNAP-25 cleavage by Western blot as described previously [26,27]. For assays using chemical stimulation of cells, cells were exposed to toxin in a modified neurobasal medium containing 80 mM KCl and 2.2 mM $CaCl_2$ (Invitrogen, custom medium) for the indicated amounts of time, followed by three washes and further incubation in NS medium.

3. Results

3.1. Neuronal cultures derived from mES cells sensitively detect BoNT/A1 activity

Mouse embryonic stem (mES) cells were differentiated to neurons by first formation of embryoid bodies (EBs) and inducing neural differentiation by treating with 0.5 μM rentinoic acid (RA) and 1 μM purmorphamine. The cells were then dissociated by gentle enzymatic treatment with acutase, and plated on laminin coated 96-well plates to allow attachment and differentiation of the neurons.

After 9 days in culture, the neurons were exposed to serial dilutions of BoNT/A1 for 24 or 48 h, and cell lysates were analyzed for SNAP-25 cleavage by Western blot (Fig. 1). After 24 h of toxin exposure, the cells consistently were exquisitely sensitive to BoNT/A1, detecting as little as 0.1 mouse LD_{50} U (Units) (104 fM), and reaching 50% SNAP-25 cleavage at about 1.6 U (1.7 pM). After 48 h of exposure, sensitivity was not increased significantly, indicating fast uptake of BoNT/A1 into the neurons. In order to further evaluate the uptake rate of BoNT/A1 into neurons, the cells were exposed to 150 U (156 pM) of BoNT/A1, and cell lysates were analyzed at 1 h time intervals for up to 6 h (Fig. 2A). A small amount of cleaved SNAP-25 was detectable as early as 2 h after toxin addition, and cleavage increased steadily over the 6 h time period and reached about 50% cleavage at 6 h. Fast and activity dependent uptake of BoNT/A1 into the cells was further confirmed by exposing the cells to 500 U (520 pM) of BoNT/A1 in stimulation medium, a modified neurobasal medium that contains 80 mM KCl and 2.2 mM $CaCl_2$ for 0, 2.5, 5, and 10 min. After exposure, the cells were washed three times to remove all residual toxin, and incubated for an additional 24 h to allow for SNAP-25 cleavage. About 70% cleavage was observed after only 2.5 min of toxin exposure, and even at the 0 min time point (toxin was added and immediately removed, followed by cell washes), significant cleavage was observed (Fig. 2B), indicating that about 30 s that elapsed between first toxin addition and the first wash of the cells was enough time for some BoNT/A1 to tightly associate with the cells. The absence of any residual toxin in the medium after the washes was confirmed by the absence of any SNAP-25 cleavage in cells exposed to this medium for 24 h (data not shown). Interestingly, significant cleavage was also observed in control cells exposed to BoNT/A1 in NS medium, although there was significantly more variation between replicate samples than when the modified neurobasal medium was used (data not shown). This indicates that the 1.8 mM $CaCl_2$ and 5.3 mM KCl contained in the basic formulation of neurobasal medium (Invitrogen) may be sufficient to support some level of neuronal cell activity.

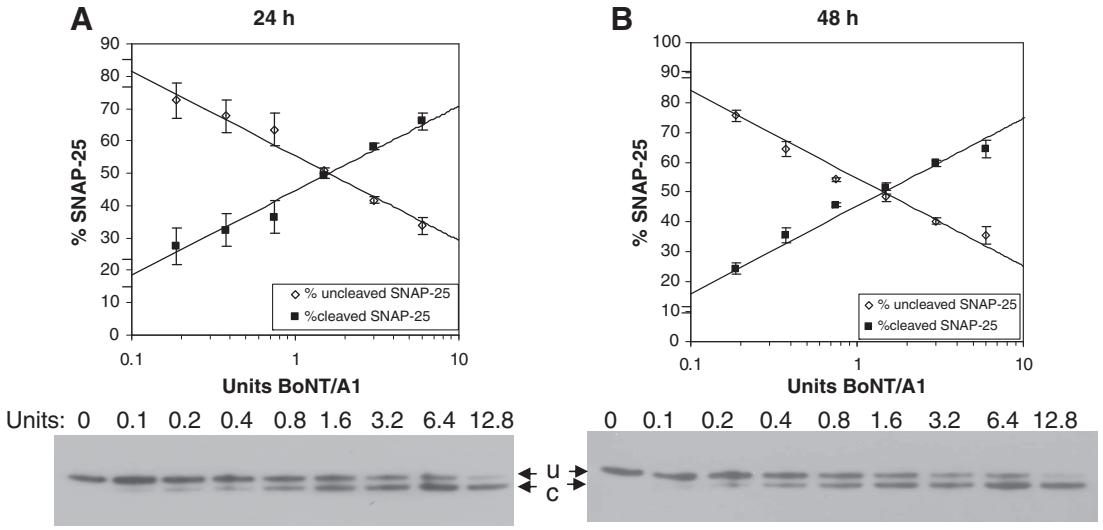

Fig. 1. BoNT/A activity in motor-neurons derived from mES cells. Mouse embryonic stem cells were differentiated to neurons, and exposed to the indicated amounts of BoNT/A for 24 h (A) or 48 h (B). BoNT/A activity was detected by analyzing cell lysates for SNAP-25 cleavage by Western blot. Densitometry data derived from three Western blots and a representative Western blot are shown. The 'u' denotes un-cleaved SNAP-25, and 'c' denotes cleaved SNAP-25.

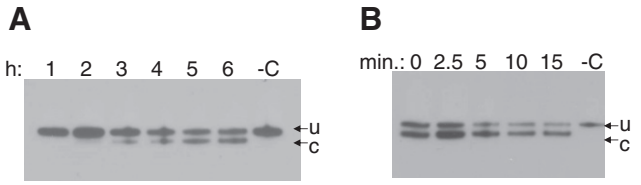

Fig. 2. BoNT/A entry kinetics into neurons derived from mES cells. Representative Western blots of triplicate experiments are shown. 'u' denotes uncleaved SNAP-25, 'c' denotes cleaved SNAP-25, and −C is cell lysates of cells not exposed to toxin. (A) The neuron cultures were exposed to 150 LD_{50} U of BoNT/A for 1–6 h, and cell lysates were analyzed for SNAP-25 cleavage. (B) The neuron cultures were exposed to 500 LD_{50} U of BoNT/A for 0–10 min in medium containing 80 mM KCl and 2.2 mM $CaCl_2$ to stimulate neuronal cell activity, followed by incubation for 24 h to allow for SNAP-25 cleavage.

3.2. Culture conditions affect BoNT/A1 sensitivity of mES derived neuron cultures

In order to examine whether small differences in the cell differentiation protocol and culture conditions affect BoNT/A1 sensitivity of the cells, cell density, RA concentration, and purmorphamine addition were varied.

A cell density of ~75,000 cells/well was chosen for most experiments because the cells produced consistent neuron cultures at that density. A higher cell density of 200,000 cells/well resulted in decreased sensitivity of the cells to BoNT/A1, with 3 U of BoNT/A1 required to reach 50% SNAP-25 cleavage (Fig. 3A). While cells seeded at densities below 50,000 cells/well resulted in inconsistent cell attachment, preliminary tests on these 'lower density' cells indicated increased BoNT/A1 sensitivity (data not shown). This is likely due to more efficient differentiation to mature neurons of the motor-neuron type at lower cell density.

Retinoic acid addition to mouse EBs in the range of 0.1–2 μM has been shown to direct motor-neuron differentiation [31,36]. In order to examine the effect of decreased RA concentration during mES cell differentiation on BoNT/A1 sensitivity of the resulting cells, the EBs were induced with 0.1 μM RA and 1 μM purmorphamine instead of 0.5 μM RA and 1 μM purmorphamine, and exposed to BoNT/A1 for 24 h. The resulting cells were at least five times less sensitive to BoNT/A1 than cells differentiated with 0.5 μM RA (Fig. 3B), indicating that even small variations in RA concentration during cell differentiation can affect BoNT/A sensitivity.

Purmorphamine is a small molecule that has been shown to efficiently induce motor-neuron differentiation of mES cells in place of the much larger protein sonic hedgehog (SHH) [31,37]. However, even in the absence of purmorphamine, RA inductions results in differentiation of about 95% of the cells to neurons, and about 5–10% to motor-neurons [31,37]. While BoNT/A1 is presumed to selectively enter motor-neurons [3,9], minimal data is available on sensitivity of other neuronal subtypes to BoNT/A1. Therefore, the requirement of purmorphamine for a cell-based BoNT/A1 assay was determined by omitting it during cell differentiation. The EBs resulting from differentiation with RA only clearly contained significantly fewer red fluorescent cells (Fig. 3D), indicating fewer HB9 expressing motor-neurons. However, the plated cells after 9 days in culture were almost indistinguishable by fluorescent microscopy from cells differentiated in then presence of purmorphamine. This could be due to either selective cell death of the more fragile differentiated motor-neurons after plating, or loss of the fluorescent signal in mature motor-neurons due to down-regulation of HB9. These cells resulted in slightly increased sensitivity to BoNT/A1, with 50% SNAP-25 being cleaved with 0.93 U (Fig. 3C). Although it is unknown whether this difference is statistically significant, these data indicate that purmorphamine is not required for the BoNT/A assay.

3.3. mES derived neuron cultures detect BoNT/E activity

The sensitivity of mES cells derived neurons to BoNT/E was evaluated by exposing the cells to serial dilutions of BoNT/E under standard conditions for 24 h. The sensitivity of the cells to BoNT/E was about five times lower than that to BoNT/A1 (Fig. 4). Decreased sensitivity of primary motor neurons to BoNT/E has also been observed [26], but the mechanisms for this difference is not known.

4. Discussion

The fast and steady progress being made in stem cell research in the past few years has opened the possibilities for the development of stem cell based toxin assays [38,39]. In particular, vast advances in the knowledge gained on differentiation of mouse embryonic

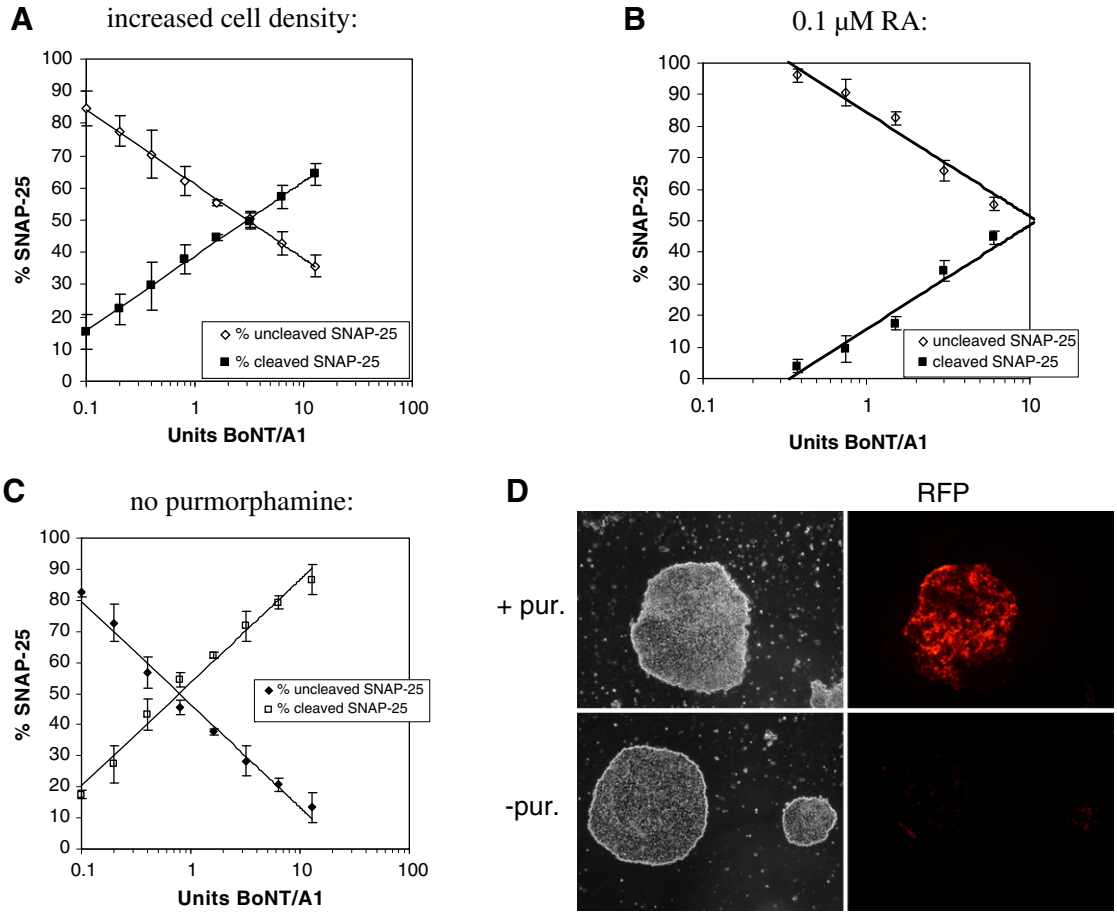

Fig. 3. Effects of the mES differentiation protocol on BoNT/A sensitivity of resulting neuronal culture. The cells were exposed to the indicated concentrations of BoNT/A for 24 h, and cell lysates were analyzed for SNAP-25 cleavage by Western blot. Densitometry data from Western blots performed in triplicate are shown. (A) The mES cells were differentiated to neurons as above, but were seeded at a 2.5-fold increased density (200,000 cells/well). (B) The mES cells were differentiated in 0.1 μM RA instead of 0.5 μM RA. (C) The mES cells were differentiated in 0.5 μM RA, but no purmorphamine was added. (D) Embryoid bodies after differentiation in the presence (+ pur) or absence (− pur) of purmorphamine. RFP fluorescence indicates motor-neurons.

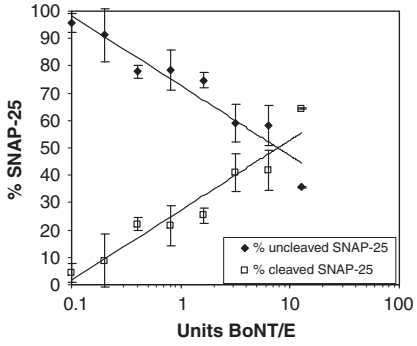

Fig. 4. BoNT/E activity in motor-neurons derived from mES cells. Mouse embryonic stem cells were differentiated to neurons, and exposed to the indicated amounts of BoNT/E for 24 h. BoNT/A activity was detected by analyzing cell lysates for SNAP-25 cleavage by Western blot. Densitometry data derived from three Western blots.

stem cells to neurons have yielded simple and fast protocols with an excellent differentiation rate [31,37]. The protocol used in this study yields about 95% of neuronal cells, of which about half can differentiate to mature motor-neurons [37]. This differentiation can easily be confirmed in the mES cell line used in this study, which expresses RFP under the control of the *HB9* promoter, which is activated in motor neurons [30,40,41]. Thus, this cell line represents an ideal system for the controlled differentiation of mES cells.

In this study, the utility of mES cell derived neuron cultures for the detection of biological activity of BoNTs was examined. Utility of cell-based assays for BoNT detection has been established [24,27], and there is a great demand for the development of a sensitive cell-based assay that does not involve cumbersome preparation of primary cells, but is equally sensitive and robust. Examination of the sensitivity of mES cell derived neurons to BoNT/A1 indicated that this cell-based testing platform can detect as little as 0.1 LD_{50} U of BoNT/A1, reaching 50% SNAP-25 cleavage at 1.6 LD_{50} U (Fig. 1). This detection limit is slightly reduced (about 5-fold) compared to primary rat spinal cord cells assay (RSC assay) [26,27], but still provides sensitivity similar to that of the MBA assay. Repetition of the assay using the same conditions yielded the same results, indicating good reproducibility. Future studies are needed to determine the inter- and intra-laboratory error of this assay.

Chemical stimulation of the cells resulted in fast uptake of BoNT/A1 within 2.5 min, which suggests that the cells neuronal activity can be activated by chemical depolarization with KCl and $CaCl_2$ (Fig. 2). Similarly fast BoNT/A1 uptake has been previously demonstrated in primary mouse spinal cord cells [42].

Variations in the differentiation protocol affected the sensitivity of the cells to BoNT/A1 (Fig. 3), even though differences in differentiation status of the plated cells were not easily apparent by fluorescent microscopy analysis. This indicates that standardization of the cell differentiation protocol and the inclusion of a toxin standard will be essential for this assay platform. The addition of purmorphamine during differentiation to induce motor-neuron

type differentiation did not result in improved sensitivity (Fig. 3C and D). However, because no clear difference could be observed by fluorescent microscopy of the plated and matured neurons at the time the assay was performed, this may be due to motor-neuron type specific cell death during culture. Future optimizations and standardizations of the cell preparation protocol will determine whether motor-neurons provide a more sensitive testing platform, and will provide the best parameters for BoNT testing.

In summary, these data suggest that neurons derived from mouse embryonic stem cells using a standardized cell preparation protocol can provide a highly sensitive cellular model for study and quantification of BoNTs. While primary cells, which are currently used in cell based BoNT detection assays, can be maintained in culture significantly longer (several months compared to about 2 weeks) allowing for longer-term studies, this assay holds several advantages over primary cells assays. These include no use of animals, no need for preparation of primary cells with the potential of lot to lot variation, the ability to standardize cell preparations, and the opportunity for inclusion of biological sensors into the cells for an easier and faster endpoint of BoNT toxicity.

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