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Model for studying *Clostridium botulinum* neurotoxin using differentiated motor neuron-like NG108-15 cells

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ABSTRACT

Cancerous cell lines have traditionally shown low sensitivity to laboratory or pharmaceutical preparations of botulinum neurotoxin. The work presented here demonstrates that the mouse neuroblastoma/rat glioma hybrid cell line NG108-15 is capable of more sensitively detecting BoNT/A1 than any cell line previously described. This cell line has previously been described to have motor neuron like characteristics, therefore making it a good model to study BoNTs. Differentiation of NG108-15 cells in serum-free medium containing retinoic acid and purmorphamine dramatically increased sensitivity of the neurons to BoNT/A (EC_{50} = ~16 LD_{50} U). Additional pre-treatment with triasialoganglioside GT1B prior to toxin exposure reduced the EC_{50} further to ~11 LD_{50} U. Co-culture of the neurons with C2C12 myotubes also significantly increased BoNT/A sensitivity of NG108-15 cells (EC_{50} = 26 U) in the absence of differentiation factors.

1. Introduction

Botulinum neurotoxins (BoNTs), synthesized by the Gram-positive, soil-dwelling bacterium, *Clostridium botulinum*, represent a unique class of bacterial virulence factors. BoNTs are the most toxic substances known to humankind and are the causative agents of botulism. Today the occurrence of botulism poisoning is rare due to improved food processing and handling procedures; however, the severity of the disease and the widespread presence and persistence of toxin-producing *C. botulinum* bacteria and spores make botulism a perennial global health concern. Additionally, BoNTs have been implicated as potential agents of biological warfare [1] and are also now widely used as pharmaceuticals for both cosmetic and clinical procedures [2].

There are seven immunologically distinct serotypes of botulinum neurotoxins (BoNTs) designated A through G [3], with some serotypes having additional subtypes (for example, BoNT/A1–/A5) [4]. All BoNTs are initially synthesized as a single-chain polypeptide of approximately 150 kDa, but posttranslational proteolytic cleavage yields distinct heavy (HC) and light chains (LC) of ~100 kDa and ~50 kDa linked by a disulfide bond. The HC is responsible for recognition, binding to specific neuronal cell surface receptors, and translocation of the LC across the endosomal membrane, which is the enzymatically active zinc-dependent endoprotease component of the toxin molecule [5–7]. The LC specifically cleaves intracellular SNARE (Soluble *N*-ethylmaleimide-sensitive factor Attachment Protein Receptor) proteins at the presynaptic nerve vesicles inhibiting the exocytosis of stimulatory neurotransmitter acetylcholine and leading to paralysis [8]. The BoNTs cleave distinct SNARE substrates depending on the serotype [8]. The experiments presented in this manuscript focused on botulinum neurotoxin type A subtype 1 (BoNT/A1), the most potent BoNT serotype and also the most frequently used as a pharmaceutical.

The symptoms of BoNT intoxication (botulism) are consistent with inhibition of primarily cholinergic neurons at the neuromuscular junctions (NMJs), which leads to a long-term, descending, flaccid paralysis [9,10]. While it is now known that BoNTs can enter other neurons [11–14], it remains largely unclear why the symptoms are primarily reflective of deactivation of cholinergic neurons. Thus, specific neuronal cell models, in particular motor neuron and neuromuscular junction (NMJ) models, are of great value in the field of botulinum research.

Several continuous cell lines (Neuro-2a, SK-N-SH, M17, SH-SY5Y, PC12 NT2) have been tested for sensitivity to BoNT/A and are being used as research models [15–24]. However, most of them lack the sensitivity necessary to compete with the commonly used mouse bioassay (MBA) [25,26], which detects low pM amounts of BoNT/A. Recent research efforts have developed protocols to optimize differentiation protocols to maximize BoNT/A sensitivity by using serum-free medium and adding neuronal growth factor (NGF) [18], retinoic acid (RA) [21], and/or addition of trisialoganglioside GT1b [15,16,19,21,24]. The most sensitive cell-based assay using a continuous cell line published to date is using the differentiated SH-SY5Y cell line, with an EC_{50} value of ~35 pM as measured

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by noradrenaline release [21]. Allergan has filed a patent for the use of SiMa cells differentiated with retinoic acid and GT1B with their product (Onabotulinumneurotoxin) with a reported sensitivity in the low pM range, comparable to the sensitivity of the MBA [24].

The NG108-15 cell line used here is a chimeric fusion of mouse neuroblastoma and rat glioma cells, and closely mimics naturally occurring motor neurons [27], which potentially makes it an excellent candidate for testing the biological activity of BoNTs. The cells are cholinergic, that is they secrete acetylcholine as their neurotransmitter. Previous work has demonstrated that NG108-15 cells co-cultured with C2C12 cells (*Mus musculus* myoblast cell line) allows for the formation of *in vitro* neuromuscular junctions (NMJs) [28–30]. Described here, the NG108-15 cell line was differentiated to form a population of cells with motor neuron-like appearances, which reliably and reproducibly detected botulinum neurotoxin with an EC_{50} value as low as ~11 mouse LD_{50} units (LD_{50} U), which is significantly more sensitive than previous cell lines tested.

2. Materials and methods

2.1. Cell lines

Cryopreserved NG108-15 (HB-12317) and C2C12 (CRL-1772) cell lines were purchased from the American Type Culture Collection (ATCC). Both cell types were thawed according to manufacturer's instructions, and maintained in 1× Dulbecco's Modified Eagle's Medium (DMEM) (CellGro) with 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen), 2% HAT supplement (Invitrogen), 100 units/mL penicillin, and 100 units/mL streptomycin (Invitrogen) at 37 °C, 5% CO_2. For differentiation, NG108-15 cells were seeded into 96-well plates (Techno Plastic Products) coated with 0.01% poly-L-ornithine (Sigma) and 8.3 μg/cm^2 Matrigel™ (BD Biosciences) at a density of ~20,000 cells per well. The cells were differentiated in serum-free medium (Neurobasal® medium supplemented with 2% B-27® Supplement, 2 mM Glutamax™, 100 units/mL penicillin/streptomycin (all from Invitrogen)). To induce motor neuron differentiation, 5 μM all-trans retinoic acid (RA) (Stemgent) and 2.5 μM purmorphamine (PUR) (CalBiochem) was added to the serum-free medium, and cells were incubated in this medium for at least 5 days with medium changes every 2 days. Where indicated, NG108-15 cells were exposed to 50 μg/mL of bovine brain trisialoganglioside G_{T1B} (SIGMA) for at least 24 h prior to BoNT/A1 toxin exposure.

2.2. Co-culture of NG108-15 cells with C2C12 myotubes

For the co-culture, C2C12 cells were seeded into 96-well plates (Techno Plastic Products) coated with 0.01% poly-L-ornithine (Sigma) and 8.3 μg/cm^2 Matrigel™ (BD Biosciences) at a density of ~200 cells per well. Cells were differentiated to myotubes for 2 days in serum-free differentiation medium prior to the addition of NG108-15 cells. The co-culture was then allowed to differentiate in serum-free medium for at least 5 days prior to use. Retinoic acid and/or purmorphamine was added as indicated.

2.3. Botulinum neurotoxin preparation

Pure botulinum neurotoxin (BoNT) serotype A (150 kDa) was prepared from *C. botulinum* strain Hall A hyper as previously described [31]. The toxin was dissolved in phosphate buffered saline (12.5 mM NaH_2PO_4, 75 mM NaCl), pH 7.4 and 40% glycerol, and stored at −20 °C until use. Activity of the BoNT/A preparation was determined by the mouse bioassay [26,32], and specific toxicity was 1.25×10^8 mouse LD_{50} U/mg.

2.4. In vitro neuronal toxicity assays

For all neuronal toxicity assays, differentiated NG108-15 cells or co-cultured C2C12/NG108-15 cells were exposed to toxin 4–8 days post-differentiation as indicated. Cells were exposed to the indicated concentrations of BoNT/A1 in 50 μL of serum-free medium. All dilutions were tested in a minimum of triplicate and a negative control without toxin was always included. After a 48 h toxin exposure, the toxin solution was removed, and cells were lysed in 50 μL of 1× lithium dodecyl sulfate (LDS) sample buffer (Invitrogen). The cell lysates were analyzed by Western immunoblot for SNAP-25 cleavage (Synaptic Systems) as previously described [33,34]. Cleaved and un-cleaved SNAP-25 bands were quantified by densitometry using a Foto/Analyst FX system and TotalLab Quant software (Fotodyne). Data plots and EC_{50} values (four parameters – variable slope) were generated using GraphPad PRISM 5 software.

2.5. Phase contrast microscopy

Differentiated and undifferentiated C2C12 and NG108-15 cells were visualized under phase-contrast using a Nikon Eclipse TE300 microscope and photographed using a Photometrics Cool SNAP HQ camera.

3. Results

3.1. Incubation in serum-free medium initiates cellular differentiation

Previous reports on NG108-15 cells differentiation described replacing the 20% FBS in the growth medium with 2% heat-inactivated horse serum [27]. However, applying this technique resulted in ~50% cell death and generated cells that required very high concentrations of BoNT/A1 to result in SNAP-25 cleavage (64,000-16,000 LD_{50} U) (data not shown). In an attempt to improve differentiation conditions of the NG108-15 cells while creating cells with the greatest sensitivity to BoNT/A1 intoxication, the normal growth medium was removed entirely, and replaced with serum-free medium. The switch from serum-rich medium (Fig. 1A) to serum-free medium reduced cell death. The NG108-15 cells took on a very distinct motor neuron-like morphology consisting of dendrites, a soma, and an axon (Fig. 1B).

3.2. Retinoic acid and purmorphamine improve cellular differentiation and minimize cell death

In order to optimize a protocol for NG108-15 cell differentiation leading to greatest BoNT/A sensitivity, several differentiation conditions were tested. This included addition of 3.8 μM nerve growth factor (NGF), 0.2 μM cAMP, 5–0.5 μM all-trans retinoic acid (RA), and/or 2.5 μM purmorphamine (PUR) to the serum-free medium, as well as different plating matrices (poly-L-ornithine (PLO), collagen, laminin, matrigel) and differentiation periods from 1–10 days. The plating matrices did not affect BoNT/A sensitivity, however, the cells appeared the healthiest on plates coated with PLO and matrigel, and thus this coating procedure was chosen as the optimal pretreatment. While addition of NGF or cAMP to the medium did not affect BoNT/A1 sensitivity, the addition of RA and PUR significantly increased sensitivity in a dose dependent-fashion, with 5 μM RA resulting in the most sensitive cells.

To further optimize differentiation conditions with RA and PUR, cells were maintained in serum-free medium alone or medium containing either 5 μM RA, or 2.5 μM PUR, or both neuronal differentiation factors together for 1–5 days. Sensitivity to BoNT/A1 was then examined by exposing the cells to 2-fold dilutions of BoNT/A

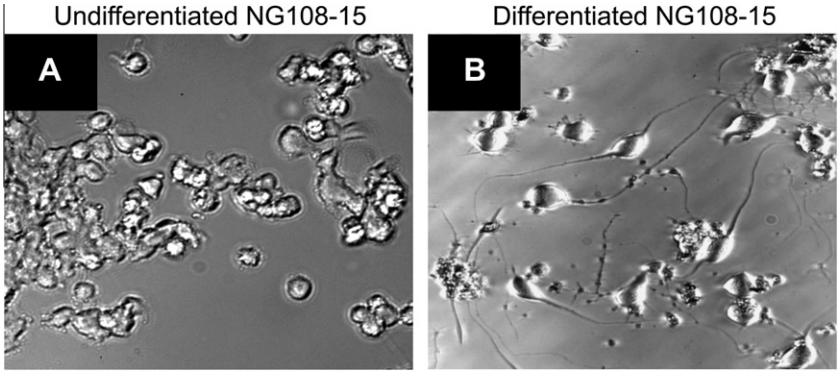


Fig. 1. Undifferentiated and differentiate NG108-15 cells. (A) Undifferentiated and (B) differentiated NG108-15 cells. Cells were differentiated in serum-free medium containing retinoic acid and purmorphamine for 5 days. Visualized under phase-contrast using a Nikon Eclipse TE300 and photographed using a Photometrics Cool SNAP HQ camera.

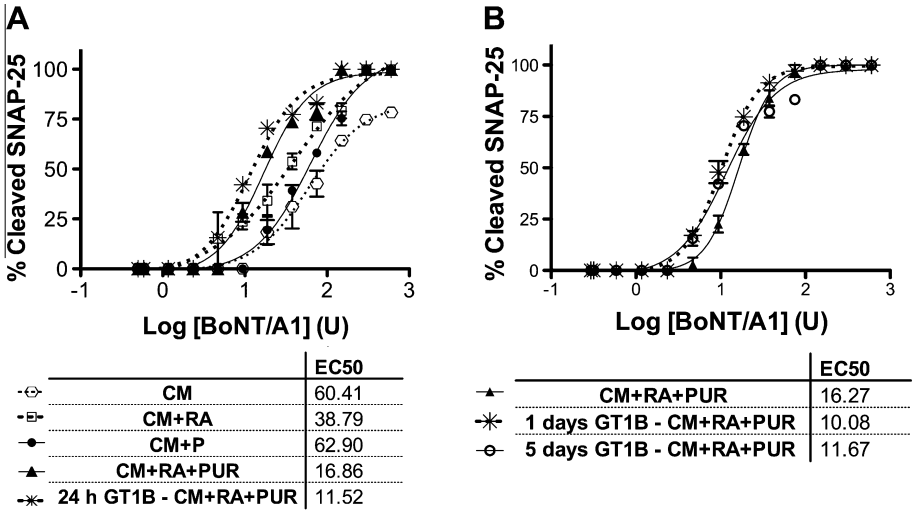
(600 LD_{50} U – 0.15 LD_{50} U) in serum-free medium and assessing SNAP-25 cleavage via Western immunoblot after 48 h. The cells differentiated in the presence of both RA and PUR for at least 5 days were most sensitive to BoNT/A, with a SNAP-25 cleavage EC_{50} value of ~17 LD_{50} U. In contrast, cells that received only RA or PUR resulted in a significantly greater EC_{50} values of ~39 LD_{50} U and ~63 LD_{50} U. Cells differentiated in only serum-free medium resulted in an EC_{50} value of ~60 LD_{50} U (Fig. 2A). Additionally, those cells differentiated in medium containing RA or PUR or both factors reached 100% cleavage of SNAP-25, while those cells in differentiation medium alone reached only ~80% cleavage at 600 LD_{50} U (Fig. 2A).

3.3. Pretreatment of NG108-15 cells with GT1b prior to toxin exposure increases sensitivity to BoNT/A1

Triasialoganglioside GT1B is a co-receptor that maximizes BoNT binding to cell surface receptors [35]. Several cell lines (PC12, Neuro-2a, SH-SY5Y) demonstrate an increased sensitivity to BoNT/A1 when pre-loaded with trisialoganglioside GT1b for 24 h prior to toxin exposure [15,16,21]. To determine if NG108-15 cells also show an increased sensitivity to BoNT/A1 after similar pre-treatment, cells previously differentiated in culture medium containing RA and PUR were exposed to 50 μg/ml of GT1b for 24 h prior to BoNT/A exposure for 48 h. GT1b-pretreated cells were ~1.5 times more sensitive to BoNT/A1 than untreated cells, with EC_{50} values of about ~12 and ~17 LD_{50} U respectively (Fig. 2A). To determine if sensitivity could be additionally increased by prolonged GT1b incubation, NG108-15 cells differentiated in medium containing 50 μg/mL of GT1b and RA and PUR for 5 days prior to BoNT/A1 intoxication, changing the medium every other day. The prolonged GT1b incubation did not further improve BoNT/A1 sensitivity (Fig. 2B).

3.4. Co-culturing NG108-15 with C2C12 cells increases NG108-15 sensitivity to BoNT/A1

To determine how the formation of *in vitro* NMJs affects NG108-15 cells sensitivity to BoNT/A1, NG108-15 cells were co-cultured with pre-formed C2C12 myotubes for 5 days in serum-free medium before intoxication (Fig. 3A). Co-cultured NG108-15 formed visible connections with C2C12 myotubes via axon and dendrite extensions after 48 h. Based on observations using light microscopy, the axon lengths of the NG108-15 cells were reduced in the co-culture as compared to the monoculture. The BoNT/A sensitivity of co-cultured cells (~37 LD_{50} U) was about 2-fold greater than that of non-co-cultured NG108-15 cells (~60 LD_{50} U) (Fig. 3B). Addition of the differentiation factors RA and PUR to the medium did not further increase the sensitivity nor improve the health of the co-cultured NG108-15 cells (~37 LD_{50} U).



	EC50
CM	60.41
CM+RA	38.79
CM+P	62.90
CM+RA+PUR	16.86
24 h GT1B - CM+RA+PUR	11.52

	EC50
CM+RA+PUR	16.27
1 days GT1B - CM+RA+PUR	10.08
5 days GT1B - CM+RA+PUR	11.67

Fig. 2. NG108-15 cell sensitivity to BoNT/A1. (A) NG108-15 cells were differentiated for 5 days in CM (serum-free differentiation culture medium), CM + RA (all-trans retinoic acid), CM + PUR (purmorphamine), CM + RA + PUR, or GT1B-CM-RA-PUR (24 h GT1B exposure) prior to BoNT/A1 addition. (B) NG108-15 cells were differentiated for 5 days in CM + RA + PUR and exposed to GT1B continuously for 5 days prior to BoNT/A1 addition.

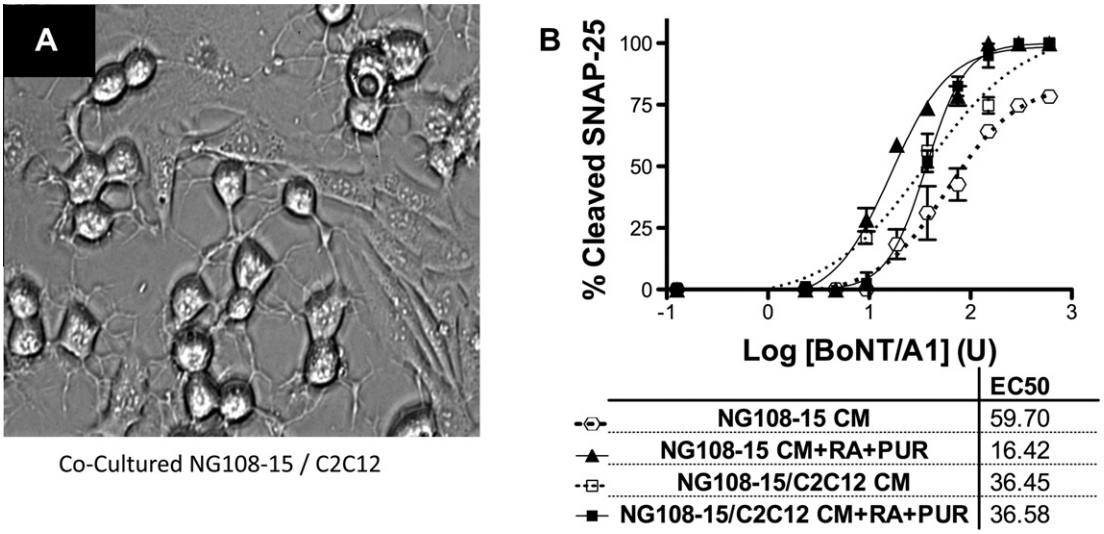


Fig. 3. NG108-15 cell sensitivity to BoNT/A1. (A) Undifferentiated NG108-15 cells were co-cultured with differentiated C2C12 cells and differentiated for 5 days. Cells were visualized under phase-contrast using a Nikon Eclipse TE300 and photographed using a Photometrics Cool SNAP HQ camera. (B) Co-cultured cells were differentiated in CM or CM + RA + PUR prior to BoNT/A1 addition. All cells were exposed to the indicated toxin dilutions for 48 in parallel. Cell lysates were analyzed for SNAP-25 cleavage by Western blot. Data from three Western blots were quantified by densitometry, and data plots and EC_{50} values were generated. The maturation time and EC_{50} values are shown in the figure legend below.

4. Discussion

As part of a screening effort to cell lines sensitive to BoNT/A, the NG108-15 cell line, a chimeric fusion of mouse neuroblastoma and rat glioma cells, with motor neuron-like characteristic was examined. Because undifferentiated NG108-15 cells are relatively insensitive to BoNT detection (EC_{50} = ~1600 LD_{50} U), the effect of differentiation of the NG108-15 cells in the serum-free medium with and without the presence of neuronal differentiation factors was monitored. These conditions led to a relatively high sensitivity (EC_{50} = ~60 LD_{50} U), however, about 50% of the cells died during this differentiation procedure, making it difficult to achieve consistent cultures of differentiated neurons. Thus, differentiation in the presence of factors known to drive motor neuron differentiation in stem cells (RA and PUR) [36–42] was examined. This increased sensitivity ~4-fold (EC_{50} = ~16 LD_{50} U). Taken together these data indicate that differentiation of NG108-15 cells in serum-free medium supplemented with 5 μM RA and 2.5 μM PUR results in consistently healthy motor neuron-like cells (Fig. 2A) with the greatest BoNT/A sensitivity.

A 24 h exposure to trisialoganglioside GT1b prior to intoxication, resulted in an additional ~1.5-fold increase in BoNT/A1 sensitivity, but a continuous exposure of 5 days did not further increase sensitivity (Fig. 2A and B). Extracellular GT1b has been shown in neuro-2a cells to be incorporated into the cell membrane, leading to a dramatic increase in BoNT/A sensitivity due to increased association of the toxin with the cell membrane [16]. Similarly, the increase in sensitivity in NG108-15 cells after GT1b incubation is likely due to an increased ability of BoNT/A to associate with the cell membrane, and therefore undergo endocytosis. While significant, the relatively small GT1b-induced increase in BoNT/A1 sensitivity can likely be explained by an increase of endogenous gangliosides as the NG108-15 cells are differentiated [43]. However, these data suggest that the cell surface becomes trisialoganglioside-saturated after the 24 h incubation such that continuous GT1B incubation does not further improve toxin binding.

Because botulism is characterized by symptoms of specific motor neuron paralysis, these cells present an interesting model system to study BoNTs. Previous reports have shown that NG108-15 cells can be differentiated into cells with motor neuron like morphologies and characteristics including the secretion of acetylcholine and the synthesis of choline acetyltransferase [44,45], the ability to readily form synapses with differentiated C2C12 cells [46], and can conduct miniature end-plate potentials (MEPPs) [47]. The BoNT/A sensitivity of such co-cultured cells (Fig. 3A) was examined, and was increased over 2-fold compared to non co-cultured cells (Fig. 3B). This increase in sensitivity is likely due to the uncharacterized, environmental factors provided by the myotubes. Surprisingly, co-culturing cells in serum-free medium containing RA and PUR did not increase sensitivity further, but this can likely be attributed to a decrease in overall cell health.

While the differentiated NG108-15 cells are ~50 times less sensitive than primary rat spinal cord (RSC) cells or stem cell derived neurons (EC_{50} ~0.3 LD_{50} U) [11,12,33,48], these cells are significantly more sensitive than cell lines previously tested. Unlike primary cells and stem cell derived neurons, these cells do not require laborious animal dissections or prolonged differentiation periods and significantly less knowledge of cellular reprogramming. Additionally, this cell line is easy to maintain, and can be robustly and consistently differentiated using the protocol described here. While pre-differentiated neurons derived from human-induced pluripotent stem cells are now available for purchase and are exquisitely sensitive to BoNTs [48], these cells are very expensive and thus not suitable for all projects involving BoNT research. Using differentiated NG108-15 cells for BoNT toxicity testing is significantly less expensive than using primary cells or hiPSC-derived neurons, and the sensitivity is sufficient for many research projects. In addition, the motor neuron like characteristics of this cell line and the ability to co-culture the neurons with myotubes to form NMJs make it an interesting BoNT study model.

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