



# Embryonic stem cell-derived neurons are a novel, highly sensitive tissue culture platform for botulinum research

Patrick McNutt<sup>a,\*</sup>, Jeremy Celver<sup>b</sup>, Tracey Hamilton<sup>a</sup>, Mariano Mesngon<sup>a</sup>

<sup>a</sup> The United States Army Medical Research Institute of Chemical Defense, APG, MD 21010, USA

<sup>b</sup> University of Rhode Island, Kingston, RI 02881, USA

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## ABSTRACT

There are no pharmacological treatments to rescue botulinum neurotoxin (BoNT)-mediated paralysis of neuromuscular signaling. In part, this failure can be attributed to the lack of a cell culture model system that is neuron-based, allowing detailed elucidation of the mechanisms underlying BoNT pathogenesis, yet still compatible with modern cellular and molecular approaches. We have developed a method to derive highly enriched, glutamatergic neurons from suspension-cultured murine embryonic stem (ES) cells. Hypothesizing that ES cell-derived neurons (ESNs) might comprise a novel platform to investigate the neurotoxicology of BoNTs, we evaluated the susceptibility of ESNs to BoNT/A and BoNT/E using molecular and functional assays. ESNs express neuron-specific proteins, develop synapses and release glutamate in a calcium-dependent manner under depolarizing conditions. They express the BoNT substrate SNARE proteins SNAP25, VAMP2 and syntaxin, and treatment with BoNT/A and BoNT/E holotoxin results in proteolysis of SNAP25 within 24 h with EC50s of 0.81 and 68.6 pM, respectively. Intoxication with BoNT/A results in the functional inhibition of potassium-induced, calcium-dependent glutamate release. ESNs remain viable and susceptible to intoxication for up to 90 days after plating, enabling longitudinal screens exploring toxin-specific mechanisms underlying persistence of synaptic blockade. The evidence suggests that derived neurons are a novel, biologically relevant model system that combines the verisimilitude of primary neurons with the genetic tractability and scalable expansion of a continuous cell line, and thus should significantly accelerate BoNT research and drug discovery while dramatically decreasing animal use.

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## 1. Introduction

The *Clostridium botulinum* neurotoxins (BoNTs) are the most poisonous substances known, with human toxicities estimated to be as low as 1–2 ng/kg [1]. Following internalization into pre-synaptic termini of neuromuscular junctions, all BoNT serotypes cleave SNARE proteins, preventing the release of neurotransmitters and inducing skeletal muscle paralysis with consequent respiratory failure [1]. Although passive immunotherapy can mitigate intoxication if administered shortly after exposure, once the toxin has entered the neuron, therapeutic options are largely limited to

supportive care [2]. Functional persistence of synaptic blockade can maintain paralysis for months, requiring sustained intensive medical care and rapidly overwhelming medical facilities in the event of an outbreak [3,4]. For these reasons, BoNTs present potent health risks, and in recognition of their disruptive potential the neurotoxins have been designated as one of six CDC ‘Category A’ bioterrorism agents.

Since the first clinical indication of BoNT poisoning is a progressive paralysis, there is a critical need for a therapeutic that facilitates recovery from synaptic blockade. Although the principle therapeutic target has been inhibition of the light chain (LC) catalytic activity, small-molecule inhibitors identified via cell-free assays have not successfully translated to cell-based or animal model systems [2,5]. Given the estimates of approximately 10–100 toxin molecules per mouse synapse after intoxication with one LD50 of BoNT/A, the law of mass action argues that a clinically effective inhibitor of catalytic activity should exhibit a  $K_i$  in the sub-nanomolar range, orders of magnitude below current candidates [6]. Conversely, while other aspects of BoNT pathogenesis such as disruption of toxin trafficking within the synapse, preservation of

Abbreviations: mESCs, mouse embryonic stem cells; ESNs, embryonic stem cell-derived neurons; LIF, leukemia inhibitory factor; BoNT, botulinum neurotoxin; PDL, poly-D-lysine; DIV, days in vitro; ATRA, all-trans-retinoic acid; ESM, embryonic stem cell medium; TEM, transmission electron microscopy; RT-PCR, reverse-transcription polymerase chain reaction; GCNs, Granule cell neurons; fSCNs, fetal mouse spinal cord neurons.

\* Corresponding author. Address: USAMRICD, 3100 Ricketts Point Road, APG, MD 21010, USA.

E-mail address: [patrick.mcnett@us.army.mil](mailto:patrick.mcnett@us.army.mil) (P. McNutt).

endplate morphology and acceleration of toxin degradation may be more amenable to therapeutic intervention, the neuronal response to intoxication and the molecular mechanisms underlying BoNT trafficking and clearance are not well understood [1,2,5]. In large part, the absence of progress in these areas can be attributed to the lack of biologically relevant, cell-based model system that is compatible with modern research and drug discovery methodologies.

Diverse cell types have been used to evaluate the effect of BoNT and candidate BoNT therapeutics on synaptic activity. Primary cultures of post-mitotic chicken, mouse or rat spinal cord cells have proven most susceptible to intoxication, with mouse and rat neurons exhibiting sensitivities to BoNT/A below 0.1 pM [7–9]. However, the use of primary neurons is limited by the necessity of dissection and isolation, complicated techniques to obtain homogeneous neuronal populations that are viable for prolonged periods, difficulty in utilizing genetic approaches and significant resource costs. An alternative strategy is utilization of a continuous cell line that can be induced to exhibit neuronal characteristics and faithfully recapitulates the full range of interactions between primary neurons and BoNT. To date, the most common of these 'neurogenic' cell lines are rat adrenal pheochromocytoma cells and human and mouse neuroblastomas. These cell lines exhibit BoNT/A EC50s (defined as the concentration of toxin that results in 50% cleavage of the target SNARE protein) in excess of 500 pM, have heterogeneous phenotypes and in most cases do not form functional synapses, making it questionable how accurately they model neuronal mechanisms of BoNT pathogenesis. Nonetheless, given the lack of alternatives, these cell-based platforms currently offer the only means to explore mechanisms of BoNT intoxication and evaluate candidate inhibitors [10–14].

An effective cell-based model system for BoNT/A research would combine the biological relevance of primary neurons with the flexibility of continuous cell lines [15]. Cells would be neuron-based, form functioning synapses that release neurotransmitter and exhibit toxin sensitivities to BoNT/A in the sub-picomolar range. They should be genetically tractable, supporting knock in, knock down and knock out experiments. Finally, cell culture should be scalable, enabling moderate-throughput screens for candidate therapeutics and large-scale systems biology approaches. Hypothesizing that murine embryonic stem-cell-derived neurons (ESNs) have the potential to satisfy these criteria, we generated highly enriched populations of ESNs from ES cells and evaluated their suitability for BoNT research and drug discovery. We report that ESNs meet these criteria and present an improved cell-based model system to identify and validate candidate therapeutic approaches.

## 2. Materials and methods

### 2.1. Cell culture

R1 ES cells (ATCC) were cultured in suspension in DMEM with 0.1 mM  $\beta$ -mercaptoethanol, 15% fetal calf serum, nonessential amino acids, L-glutamine and 1000 units/mL LIF. Cell aggregates were dissociated with TrypLE (Invitrogen) every 48 h and subpassaged to  $1.5 \times 10^5$  cells/mL. Neurons were derived between passages 5 and 30 similar to published methods [16,17], with the differences that differentiating aggregates were maintained in low attachment petri dishes (Corning) on a rotary shaker at 45 rotations per min. Cell aggregates were dissociated with TrypLE and plated in N2 medium (Neurobasal-A medium with L-glutamine and N-2 supplement; Invitrogen) at  $1 \times 10^5$  cells/cm<sup>2</sup> in PDL (Sigma)-coated tissue culture dishes. Cells were washed with N2 medium at 2 and 24 h and subsequently cultured in B27 med-

ium (Neurobasal-A medium with L-glutamine and B27 supplement; Invitrogen) starting at DIV 2. For transgenesis, ES cells were transfected with a construct expressing mVenus under control of the CAG promoter using Lipofectamine (Invitrogen). Clonal aggregates were expanded and differentiated after 10 days of selection with 250  $\mu$ g/mL geneticin (Invitrogen) [18]. GCNs (Lonza) were cultured according to vendor's directions. BoNT/A holotoxin and BoNT/E protoxin were purchased from Metabionics; BoNT/E protoxin was activated by nicking [8].

### 2.2. RT-PCR and fluorescent immunoblots

Total RNA was isolated from GCNs or ESNs using RNeasy (Qiagen) and reverse-transcribed to cDNA (Superarray). Transcripts were amplified in a Biorad CFX96 with 30 cycles of: 30 s at 94 °C, 20 s at 60 °C, and 30 s at 72 °C and detected by separation on a 2% agarose gel using primers in Supplemental Table 1. For western blots, ESNs were harvested in RIPA buffer (Sigma). Fifteen micrograms of total protein was separated on a 12% SDS-PAGE NuPage gel (Invitrogen) and transferred to PVDF membranes. Blots were probed per Supplemental Table 2.

### 2.3. HPLC and fluorometric detection of basal and evoked release of primary amines

Basal electrophysiologic buffer (BEB; 5 mM HEPES, 10 mM glucose, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 2 mM KCl and 121 mM NaCl) and high potassium electrophysiologic buffer (KEB; BEB with 53 mM NaCl and 70 mM KCl) were adjusted to 290 mOsm with NaCl. ESNs were washed three times and incubated for 3 min at 37 °C in prewarmed BEB. Supernatant was collected and immediately replaced with prewarmed KEB for an additional 3 min at 37 °C. Primary amines in each supernatant were derivatized with *o*-phthalaldehyde (Pierce), separated by reverse-phase HPLC on an Eclipse Plus C18 column (2.1  $\times$  50 mm; Agilent) and quantified by fluorometry [19]. The linear response of this assay was determined to be between 500 fM and 100 pM. For evaluation of calcium-dependent exocytosis, cells were incubated in 50  $\mu$ M BAPTA (Invitrogen) in B27 medium for 30 min prior to analysis.

### 2.4. Cell imaging

ESNs plated on 18 mm PDL-coated coverslips (Sigma) were fixed for 15 min in 3.7% formaldehyde, permeabilized with 0.1% saponin in PBS and blocked with 3% BSA. Primary antibodies (Supplemental Table 2) were applied for 1 h at RT, followed by Alexa-conjugated secondary antibodies (Invitrogen). Coverslips were mounted with Prolong Gold DAPI (Invitrogen) and imaged using a Zeiss confocal microscope. In some instances, z-stacks were converted to a maximum projection image and deconvolved using Zen (Zeiss) software. To visualize GFP expression, cells were fixed and mounted. For assessment of cell viability, ESNs were incubated in 2  $\mu$ M calcein (Invitrogen) in Neurobasal-A medium at 37 °C for 20 min and imaged.

## 3. Results

### 3.1. Derivation of neurons from suspension-cultured ES cells

Murine ES cells maintained in feeder cell co-culture are competent to differentiate into neurons [16,17]. We first asked whether the more economic and technically simpler method of suspension culture without feeder cells or conditioned medium was compatible with neurogenesis (Supplemental Fig. 3). We recovered  $9.8 \pm 2.1 \times 10^7$  neural progenitor cells (NPCs) at DIV 0,  $69 \pm 9\%$  of

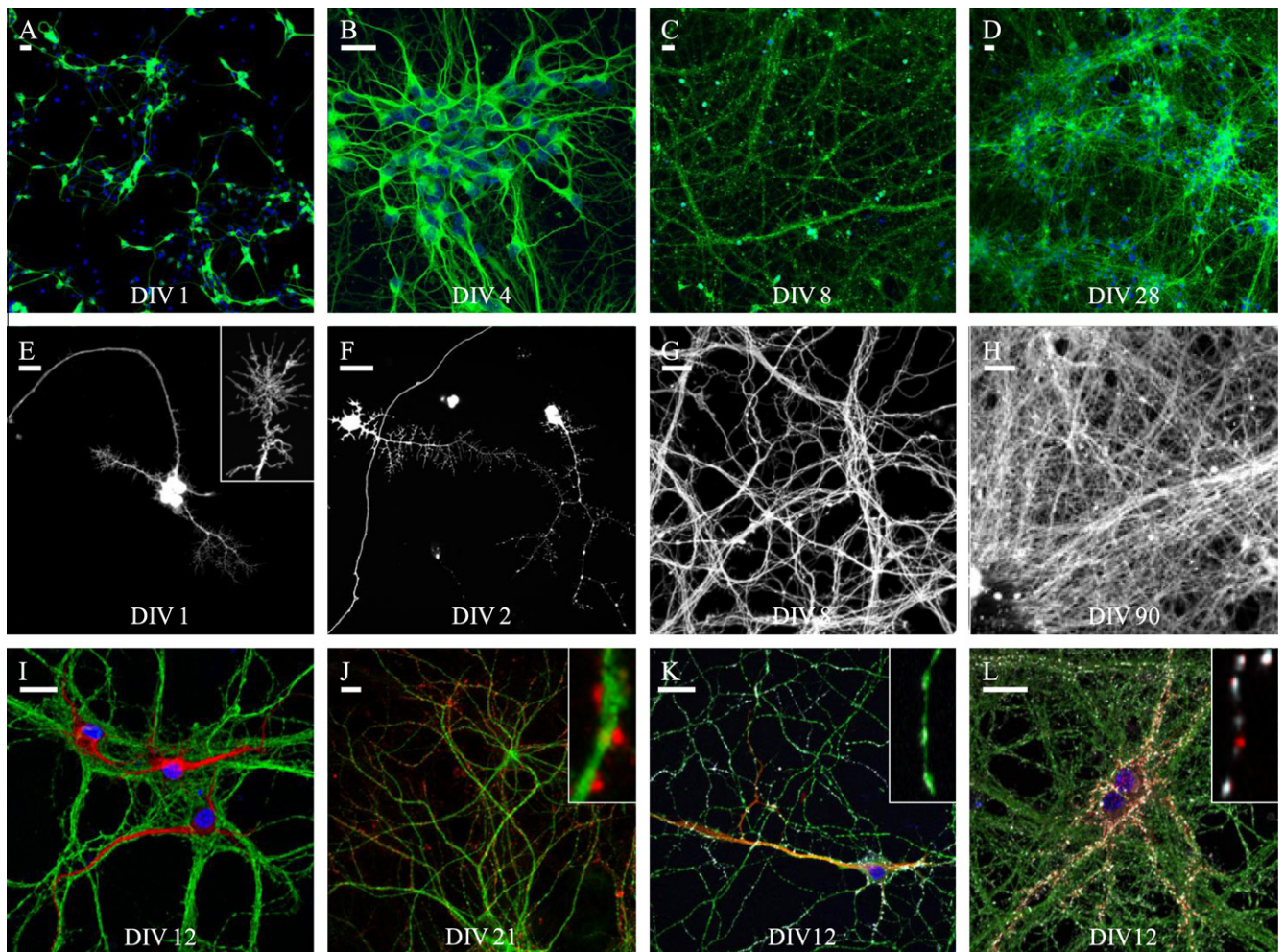
which were positive for the neuron-specific marker  $\beta$ 3-tubulin at DIV 1 (Fig. 1A;  $n = 30$ ). This is a 2.7-fold improvement over previous reports of neuronal yield [20]. Following a series of washes in serum-free media to select against non-neuronal cell types and facilitate neurite extension, by DIV 4 approximately 98% of surviving cells were positive for  $\beta$ 3-tubulin (Fig. 1B).

ESNs develop articulated neurites soon after plating (Fig. 1B and G) and a dense axodendritic arbor is apparent by DIV 12 (Fig. 1I, K, and L). The significant increase in length of MAP2-positive processes between DIV 12 and 21 (compare Fig. 1I–K) demonstrates the continued development of the neural network through at least 3 weeks. Incubation with the vital marker calcein indicates that ESNs remain viable through DIV 90 (Fig. 1E–H), suggesting that cultures may be suitable for long-term characterization of toxin susceptibility and persistence. ES cells were stably transfected to express YFP under control of the synthetic CMV: $\beta$ -actin promoter and seven clones were picked, expanded and differentiated. All

clones continued to fluoresce through DIV 28 and no differences in neurogenic potential or yield were observed (Fig. 1C and D).

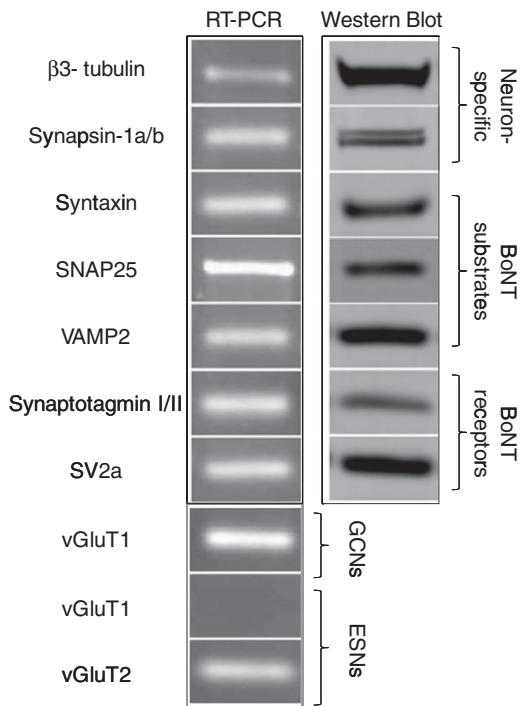
### 3.2. ESNs form synapses and express proteins necessary for BoNT activity

Consistent with the increase in arborization during the first 3 weeks, ESNs displayed histological characteristics of neuronal maturation, including segregation of the axonal marker Tau and the dendritic/somal marker MAP2 (Fig. 1I); localization of the pre-synaptic markers synapsin and vesicular glutamate transporter isoform 2 (vGluT2) to tau-reactive processes (Fig. 1J and K); and apposition of the pre-synaptic marker synaptophysin and the post-synaptic marker PSD95 (Fig. 1L). Greater than 96% of cells expressed the glutamatergic marker vGluT2, less than 2% were immunoreactive for GAD67 (a gabaergic marker) and markers for cholinergic (HB9 and ChAT), dopaminergic (GATA) or serotonergic



**Fig. 1.** Immunofluorescent characterization of derived neurons. (A and B)  $\beta$ 3-Tubulin (green: early neuron-specific marker) and nuclear (blue) staining of ESNs at DIV 1 (A) and DIV 4 (B) demonstrates enrichment for neurons by serum-free selection against non-neuronal cell types over the first 4 days. (C and D) Transgenic ES cells were differentiated into ESNs and eGFP expression (green) with a nuclear counterstain (blue) was imaged from a representative clone at DIV 8 (C) and DIV 28 (D). Note the sustained eGFP expression even 4 weeks after plating. (E–H) Live cell images of calcein-stained ESNs at DIV 1 (E), DIV 2 (F), DIV 8 (G) and DIV 90 (H). The axodendritic arbors increase in complexity and density of as a function of time in culture. (I–L) Visualization of markers of neuronal maturation and synaptogenesis. (I) Partitioning of the dendrite/soma-specific marker MAP-2 (red) and the axon-specific marker Tau (red) is occurring in DIV 12 ESNs. Nuclei are labeled in blue. (J) Expression of axonally-localized vGluT2 (red) and dendritic MAP-2 (green) in DIV 21 ESNs. Note in the inset that vGluT2 abuts rather than overlaps the dendritic compartment. Also note the increased length and branching of dendrites at DIV 21 versus DIV 12 (Fig. 1I). This increase in dendritic complexity was routinely observed. (K) Four-color immunofluorescence characterizing expression of the pre-synaptic marker synapsin (white), the dendritic marker MAP-2 (red), the axonal marker Tau (green) and cell nuclei (blue). The inset demonstrates co-localization of the synapsin within the axon, in contrast to the inset in 2J. (L) Four-color immunofluorescence characterizing expression of the pre-synaptic marker synaptophysin (white), post-synaptic marker PSD95 (red), the axonal marker Tau (green) and nuclei (blue). The inset demonstrates close proximity between synaptophysin and PSD95, which is characteristic of mature synapses. Scale bar is 20  $\mu$ m in all panels.





**Fig. 2.** Derived neurons express genes consistent with neuron derivation, maturation and susceptibility to intoxication. (Left) RT-PCR for β3-tubulin (early neuronal marker); synapsin-1a/b (marker of synaptogenesis); the proteolytic targets of the different BoNT serotypes (SNAP25, VAMP2 and syntaxin-1a); and the presumptive BoNT receptors SV2a and synaptotagmin I/II. (Middle) Immunoblot analysis for the same markers. (Right) RT-PCR showing that ESNs express vGluT2 but not vGluT1. As a positive control, vGluT1 transcript was detected from DIV 8 GCNs.

(SERT) neuronal phenotypes were not observed. TEM micrography is consistent with immunofluorescent evidence of synaptogenesis, demonstrating the assembly of characteristic synaptic architectures, including the presence of a synaptic cleft, synaptic vesicles in the pre-synaptic compartment and post-synaptic ribosomal densities (Supplemental Fig. 4).

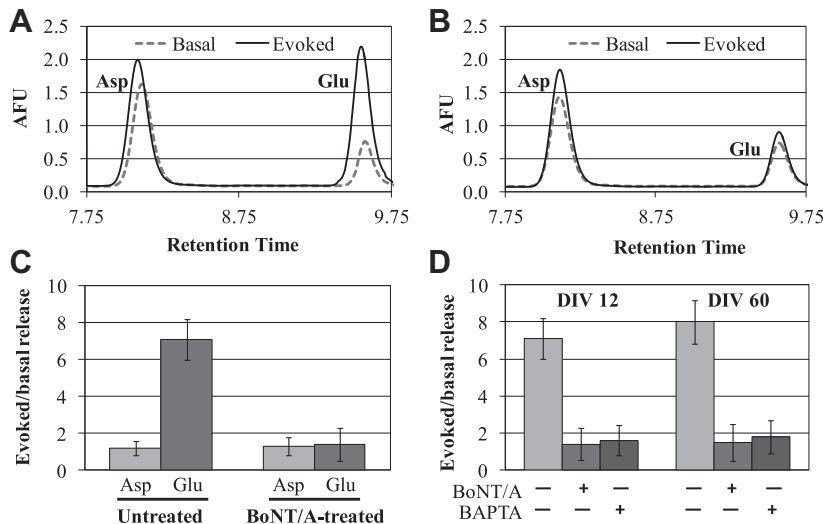
ESNs express the three BoNT target proteins SNAP25, VAMP2 and syntaxin-1 as soon as DIV 5 (Fig. 2). Functionally these three SNARE proteins assemble into a ternary complex that mediates calcium-dependent synaptic exocytosis. Expression of pre-synaptic membrane BoNT receptors SV2 and synaptotagmin further supports the hypothesis that derived neurons contain the necessary machinery for BoNT pathogenesis and synaptic activity. Finally, the transcript for vGluT2, but not vGluT1, was expressed in ESNs.

**3.3. Calcium-dependent, glutamate exocytosis is inhibited by BoNT/A treatment**

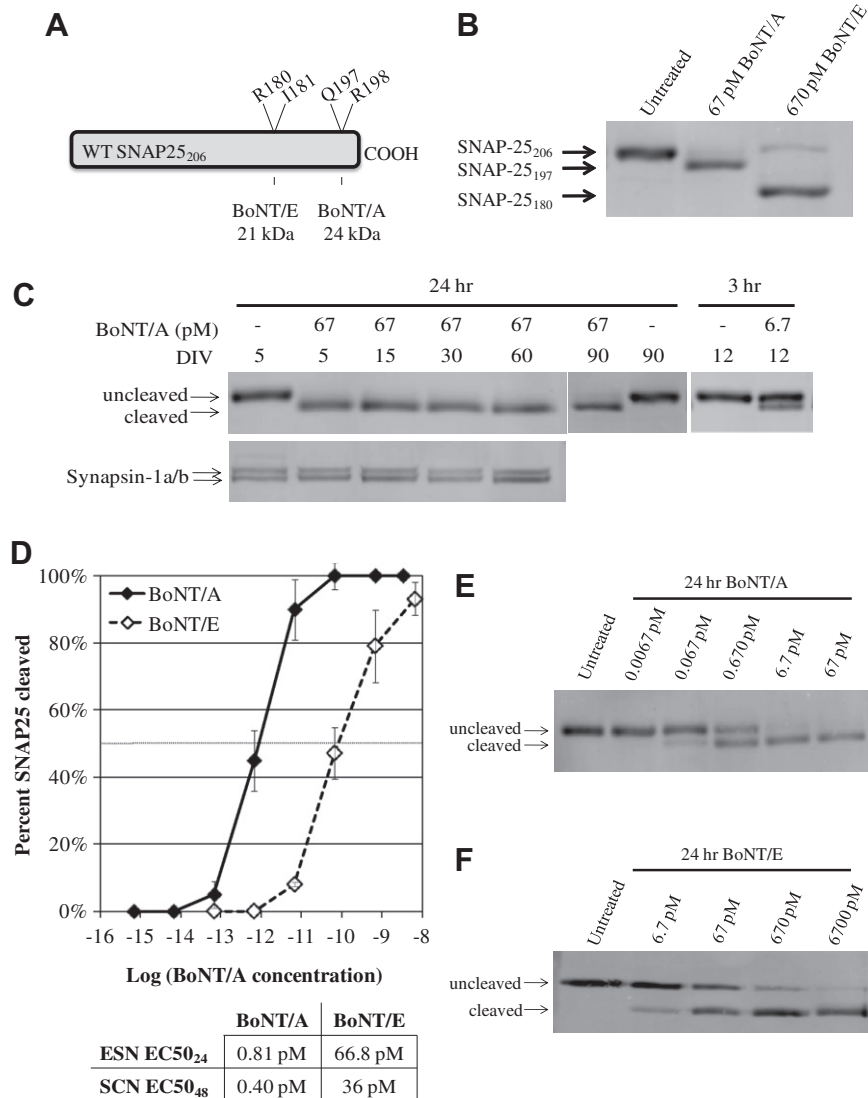
The competency of ESNs to undergo synaptic exocytosis under depolarizing conditions was evaluated by HPLC. Similar to primary neuron cultures, ESNs constitutively release several amino acids, including glycine, aspartate and glutamate [21,22]. Exposure of ESNs to high-potassium specifically elicited a 700% increase in extracellular glutamate without significantly altering the release of other primary amines (Fig. 3A and B). This is consistent with data suggesting the majority of ESNs are glutamatergic. To demonstrate that potassium-induced glutamate release is calcium-dependent, ESNs incubated with an intracellular calcium chelator did not undergo potassium-evoked glutamate release (Fig. 3C). Treatment of ESNs with 67 pM BoNT/A for 24 h prevented potassium-evoked glutamate release (Fig. 3B and D).

**3.4. ESNs are highly sensitive to both BoNT/A and BoNT/E**

Cleavage of SNAP25 by BoNT/A or BoNT/E generates a proteolytic fragment that can be distinguished from uncleaved SNAP25 by gel electrophoresis (Fig. 4A). Consistent with the neurotransmitter release assay, treatment of ESNs with 67 pM BoNT/A or 670 pM BoNT/E for 24 h resulted in near-complete cleavage of SNAP25 (Fig. 4B). ESNs were susceptible to a 24 h treatment with 67 pM BoNT/A between DIV 5 and 90, demonstrating a prolonged time-frame during which therapeutics may be evaluated (Fig. 4C). To more clearly characterize the sensitivity of ESNs to BoNT/A and BoNT/E, we determined the concentration of BoNT holotoxin that caused 50% cleavage of cellular SNAP25 within 24 h (EC<sub>50</sub><sub>24</sub>). The



**Fig. 3.** Treatment of ESNs at DIV 12 inhibits calcium-dependent neurotransmitter release. HPLC was used to quantify neurotransmitter release from derived neurons in basal and stimulatory buffers. (A and B) Representative chromatograms from DIV 12 untreated (A) and BoNT/A-treated (B) ESNs showing that BoNT/A treatment specifically inhibits Glu release in the presence of high potassium. (C) Potassium treatment specifically evokes a 700% increase in extracellular Glu, but not Asp. This increase in potassium-evoked Glu release is completely inhibited by 67 pM BoNT/A. (D) Preincubation of DIV 12 or 60 ESNs in BoNT/A or BAPTA/AM blocks potassium-evoked glutamate release. Vertical axis represents relative concentration of evoked over basal glutamate release in untreated ESNs. Values represent areas under the curve and  $n = 3$  for all data points.



**Fig. 4.** ESNs are susceptible to intoxication by BoNT/A and BoNT/E. (A) Cartoon demonstrating cleavage sites for BoNT/A and BoNT/E. (B) Representative immunoblot demonstrating that exposing ESNs to either BoNT/A or BoNT/E for 24 h results in near-complete proteolysis of SNAP25. (C) Left: Representative immunoblots demonstrating that exposing ESNs to BoNT/A for 24 h between DIV 5–90 results in complete cleavage of SNAP25. Some samples were co-stained for synapsin 1a/b as a loading control; note that SNAP25 expression did not decrease as a fraction of total protein during the first 60 days in culture. Right: Treatment of ESNs for 3 h with 6.7 pM BoNT/A followed by harvest results in cleavage of 25% of SNAP25. (D–F) Dose response of DIV 12 ESNs to BoNT/A and BoNT/E. (D) Log:lin plot calculated from densitometric measurements of SNAP25 cleavage after exposing ESNs to 0–67 pM BoNT/A ( $n = 6$ ) or 0–6.7 nM BoNT/E ( $n = 5$ ) for 24 h. The table below the graph summarizes comparative BoNT/A and BoNT/E EC<sub>50</sub><sub>24</sub>s. (E) Representative immunoblot showing typical dose–response data for BoNT/A treatment. (F) Representative immunoblot showing typical dose–response data for BoNT/E treatment.

BoNT/A EC<sub>50</sub><sub>24</sub> was calculated to be 0.81 pM (Fig. 4D and E). In comparison, fSCNs have an EC<sub>50</sub><sub>48</sub> of 0.4 pM, while neurogenic cell lines are 2–3 orders of magnitude less sensitive [8,9,23]. The BoNT/E EC<sub>50</sub><sub>24</sub> was 66.8 pM, as compared to a fSCN EC<sub>50</sub><sub>48</sub> of 36 pM (Fig. 4D and F) [8]. Finally, a 3 h exposure to 67 pM BoNT/A resulted in cleavage of 25% of SNAP25 (Fig. 4C).

#### 4. Discussion

A common technique in drug discovery is the use of cell-based models to identify targets, conduct primary or secondary screening of candidate libraries and validate hits before translation to animal models. Although cell-free systems are frequently used to screen for inhibitors of BoNT light chains, candidate drugs often fail during subsequent cell- or animal-based validation due to incompatibilities with biological assays [2,5]. Discrepancies between *in vivo* assays and neurogenic cell lines in BoNT inhibitor efficacy studies

further demonstrates the critical need for a biologically relevant, cell-based model system that can serve as the foundation of a drug discovery program [14]. Hypothesizing that ES cell-derived neurons might satisfy this requirement, we developed a method to generate large numbers of enriched glutamatergic neurons from suspension-cultured ES cells. Using immunological, ultrastructural and molecular analyses we found that ESNs assume a neuronal morphology, express neuron-specific proteins, form synapses and release glutamate in a calcium-dependent manner under depolarizing conditions.

The high degree of sensitivity of ESNs to BoNT/A and BoNT/E holotoxins suggests that ESNs support efficient toxin binding, translocation and SNAP25 cleavage [8]. This is further corroborated by the rapid uptake and activation of BoNT/A, which is indicative of targeted synaptic endocytosis of the toxin as opposed to internalization and axolemma diffusion via non-specific endocytosis [24]. The prolonged susceptibility of derived neurons to BoNT/A intoxi-

cation will allow for exploration of mechanisms underlying toxin persistence and synaptic recovery, as well as for screens to identify pharmacologics that rescue neurotransmission in intoxicated neurons.

The development of a biologically relevant, genetically tractable model system is critical for BoNT research and drug discovery [15]. Stable transfection of ES cells does not appear to interfere with neurogenic potential, and ESNs express recombinant proteins for at least 4 weeks after plating. The ability to create large numbers of transgenic neurons avails BoNT researchers with new approaches, such as reverse genetics to determine the role(s) of proteins in intoxication and synaptic recovery; identification of therapeutic targets by knock-down screens; application of fluorescent reporters to evaluate toxin activity; and utilization of moderate-throughput techniques to screen for BoNT inhibitors. While the CAG promoter sustains transgene expression following differentiation, tissue-specific promoters could also be used to provide additional control over the timing and magnitude of transgene expression [26,27]. The development of transgenic stem cell lines may be streamlined by isolation and expansion of ES cells or induced pluripotent stem cells from transgenic mice, allowing the comparison of *in vivo* and *in vitro* findings.

The development of ESNs resolves problems with cost and reproducibility that have hindered moderate-throughput drug screening approaches based on primary neurons and neurogenic cells. Unlike neurogenic cell lines, ESNs form homogenous neuron cultures which respond to toxin in a sensitive and reproducible manner. The high degree of purity and scalable derivation enables the use of 'omics' approaches to evaluate short- and long-term cellular responses to intoxication, and makes moderate-throughput drug screening for BoNT inhibitors viable. In terms of risk management, ESNs are intoxicated by 100–1000-fold less toxin than other neurogenic cell lines, reducing the dangers of toxin handling and enabling the purchase, storage and use of much smaller quantities of BoNT/A. These same characteristics also allow for the economic utilization of ESNs as a potential animal use replacement in mouse LD50 bioassays to quantify toxin potency and antitoxin efficacy [25].

These data suggest that ESNs comprise a novel cell model for BoNT research that combines the verisimilitude of primary neurons with the flexibility of continuous cell lines. The identification of a robust, scalable, genetically tractable and sensitive neuron-based research platform should enable detailed biochemical and molecular approaches that are not feasible in neurogenic cells or primary neurons. We anticipate this platform will expedite the identification and validation of novel therapeutic approaches to BoNT intoxication as well as the elucidation of intracellular aspects of pathogenesis.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bbrc.2010.12.132](https://doi.org/10.1016/j.bbrc.2010.12.132).

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