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REGULAR ARTICLE

Embryonic stem cell-derived motoneurons provide a highly sensitive cell culture model for botulinum neurotoxin studies, with implications for high-throughput drug discovery☆

Erkan Kiris[a,b], Jonathan E. Nuss[a], James C. Burnett[c,d], Krishna P. Kota[a], Dawn C. Koh[b], Laura M. Wanner[a], Edna Torres-Melendez[a], Rick Gussio[d], Lino Tessarollo[b], Sina Bavari[a,*]

[a] *Department of Target Discovery and Experimental Microbiology, US Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Frederick, MD 21702–5011, USA*
[b] *Neural Development Group, Mouse Cancer Genetics Program, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702, USA*
[c] *SAIC Frederick, Inc., Target Structure-Based Drug Discovery Group (TSBDDG), National Cancer Institute at Frederick, MD 21702, USA*
[d] *TSBDDG, Information Technology Branch, Developmental Therapeutics Program, National Cancer Institute at Frederick, MD 2170, USA*

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Abstract Botulinum neurotoxins (BoNTs) inhibit cholinergic synaptic transmission by specifically cleaving proteins that are crucial for neurotransmitter exocytosis. Due to the lethality of these toxins, there are elevated concerns regarding their possible use as bioterrorism agents. Moreover, their widespread use for cosmetic purposes, and as medical treatments, has increased the potential risk of accidental overdosing and environmental exposure. Hence, there is an urgent need to develop novel modalities to counter BoNT intoxication. Mammalian motoneurons are the main target of BoNTs; however, due to the difficulty and poor efficiency of the procedures required to isolate the cells, they are not suitable for high-throughput drug screening assays. Here, we explored the suitability of embryonic stem (ES) cell-derived motoneurons as a renewable, reproducible, and physiologically relevant system for BoNT studies. We found that the sensitivity of ES-derived motoneurons to BoNT/A intoxication is comparable to that of primary mouse spinal motoneurons. Additionally, we demonstrated that several BoNT/A inhibitors protected SNAP-25, the BoNT/A substrate, in the ES-derived motoneuron system. Furthermore, this system is compatible with immunofluorescence-

Abbreviations: ES, embryonic stem; RA, retinoic acid; Shh, sonic hedgehog; EBs, embryoid bodies; WT, wild type; FACS, fluorescence-activated cell sorter.

☆ The views, findings, interpretations, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Department of Health and Human Services or the U.S. Army.

* Corresponding author. Fax: +1 301 619 2290.
E-mail address: sina.bavari@amedd.army.mil (S. Bavari).

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based high-throughput studies. These data suggest that ES-derived motoneurons provide a highly sensitive system that is amenable to large-scale screenings to rapidly identify and evaluate the biological efficacies of novel therapeutics.
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Introduction

Botulinum neurotoxins (BoNTs), composed of seven biochemically distinct serotypes (BoNT/A–G) secreted by anaerobic bacteria *Clostridium botulinum*, are extremely potent inhibitors of neurotransmitter exocytosis at neuromuscular junctions (Neale et al., 1999; Rossetto et al., 2006; Montecucco and Molgo, 2005). Furthermore, BoNTs have been weaponized (Arnon et al., 2001), and there is a significant concern that one or more of these toxins could easily be used during an act of bioterror (Wein and Liu, 2005). Indeed, BoNTs, as the most poisonous of known bacteria toxins (Lamanna, 1959), are listed as category A bio-threat agents by the Centers for Disease Control and Prevention (Centers for Disease Control and Prevention, 2010).

On the other hand, and despite their unmatched toxicities, pharmaceutical grade BoNT serotypes A and B are widely used, in quantitatively minute, localized doses: (i) to treat various movement and hyperactivity disorders and (ii) cosmetically for wrinkle reduction (Jankovic, 2004; Dolly et al., 2009; Brin, 2009). In fact, Botox (BoNT/A) treatment is FDA approved and, according to statistics provided by the American Society for Aesthetic Plastic Surgery (American Society for Aesthetic Plastic Surgery, 2010), is one of the top nonsurgical cosmetic procedures performed in the United States. Furthermore, to extend BoNT potency for clinical neurology applications, chimeric and modified BoNTs have been generated using protein engineering methods to expedite the toxin's cellular entry, increase its substrate specificity, and extend its duration of activity in neuronal and possibly nonneuronal cells (Dolly et al., 2009; Chen and Barbieri, 2009; Wang et al., 2008; Foster et al., 2006).

However, as BoNTs are increasingly being used for medical and cosmetic purposes, the possibility of accidental overdosing in the clinic, in addition to unintentional environmental exposure through contaminated food or liquids, is also increasing. Moreover, as indicated above, BoNTs represent a major concern with regard to counter-bioterrorism efforts. Yet, there is still no effective therapeutic means for countering these toxins postneuronal internalization (Montecucco and Molgo, 2005). To date, there are only two FDA approved anti-BoNT antibodies: babyBIG (human serum-derived anti-BoNT immunoglobulins) and bivalent (BoNT A/B) equine toxin (Infant Botulism Treatment and Prevention Program, 2010; Larsen, 2009). However, the antibody treatments must be administered to intoxicated individuals within a very narrow time frame to prevent the progression of neuronal paralysis—since the antibodies cannot inhibit the toxins postneuronal penetration. As a result, the antibodies are only marginally beneficial for recovery, since patients still require respiratory aid for weeks. Hence, there is an urgent need to develop novel and more effective medical treatments to limit/counter BoNT intoxication (Burnett et al., 2005).

Mechanistically, BoNTs inhibit neurotransmitter exocytosis by cleaving specific soluble *N*-ethylmaleimide-sensitive fusion protein attachment (SNARE) proteins. The cleavage of SNARE proteins results in impaired muscle function, respiratory arrest, and, if mechanical respiration is not available, death (Neale et al., 1999; Baldwin and Barbieri, 2009; Rossetto et al., 1994). Structurally, BoNTs are composed of a heavy chain (HC) (100 kDA), which is mainly responsible for neuronal internalization, and a light chain (LC) metalloprotease (50 kDa) (Grumelli et al., 2005; Simpson, 2004). The HC and the LC are linked by a disulfide bridge (Montecucco and Molgo, 2005). The C-terminal region of the BoNT HC interacts with SV2 (Synaptic Vesicle Protein 2) receptors, and mediates toxin endocytosis (Dong et al., 2006; Mahrhold et al., 2006). Following cellular entry, the LC dissociates from the HC and cleaves SNARE proteins in the neuronal cytosol (Rossetto et al., 2006). Importantly, it is the LC's proteolytic activity that is responsible for the extreme potencies of these toxins; LC at very low concentrations is sufficient to block the activity of a motoneuron. For example, BoNT/A specifically removes 9 amino acids from the C-terminus of SNARE component synaptosomal-associated protein of 25 kDA (SNAP-25), and this simple cleavage is sufficient to inhibit neurotransmitter release (Kalandakanond and Coffield, 2001; Apland et al., 1999, 2003; Blasi et al., 1993; Bajohrs et al., 2004). Moreover, while BoNT/A mainly targets motoneurons, it is stable in cells for weeks (Adler et al., 2001; Keller et al., 1999; Fagan et al., 2009), and its active form can travel in the nervous system and reach distant synapses through retrograde transportation (Caleo et al., 2009; Antonucci et al., 2008).

There is a significant need to develop novel therapeutics to counter BoNT poisoning—especially postneuronal internalization (Hakami et al., 2010). However, there are currently no reliable, large-scale cell-based drug screening assays to facilitate such research. This is mainly due to a lack of relevant and well-characterized experimental model systems. Specifically, while cell culture-based assays employing mammalian neuroblastoma cells and primary spinal cord cells from chickens or rodents have been used previously to study the effects of small molecules on BoNT intoxication (Hakami et al., 2010; Sheridan et al., 2005; Coffield and Yan, 2009; Stahl et al., 2007; Dong et al., 2004; Pellett et al., 2007), these models possess limitations. For example, immortalized cells can be produced in large quantities, but lack sensitivity (Hakami et al., 2010). Moreover, such cell lines carry tumorigenic properties, and therefore do not mimic physiologically normal/relevant conditions. In contrast, primary embryonic spinal cord cells from rodents are physiologically relevant and very sensitive to BoNT intoxication (Keller et al., 2004); however, these cells are very difficult to obtain in large quantities because primary embryonic spinal motoneurons are postmitotic. Moreover, spinal cord dissections are technically challenging and inconsistent, as they can yield different ratios of motoneurons (versus other cells) from culture to culture. Therefore, these strategies are not optimal for developing high-throughput, neuron-based screening assays.