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# Suitability of Clonal NS26 Cells for Botulinum Neurotoxin Studies

Heather Manley
Heidi Hoard-Fruchey
Michael Adler

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**US Army Medical Research Institute of Chemical Defense**
**8350 Ricketts Point Road**
**Aberdeen Proving Ground, MD 21010-5400**

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We examined the NS-26 neuroblastoma cell line for suitability as a model for BoNT investigation. These cells are derived from a sympathetic ganglion tumor and have been reported to synthesize, store and release ACh and to form cholinergic synapses with skeletal muscle. In the current study, NS-26 cells were found to express the SNARE proteins SNAP-25 and syntaxin and to release ACh in a voltage- and Ca2+-dependent fashion. ACh release was assessed by the uptake and release of radiolabeled ACh or by the quenching of the fluorescent dye FM1-43. For qualitative measure of synaptic activity, the latter was found to be as reliable as the radioisotope method but was more rapid and required less sample manipulation. Exposure of NS-26 cells to 10 nM BoNT/A resulted in complete inhibition of evoked ACh release accompanied by cleavage of ~35% of SNAP-25. NS-26 cells were also sensitive to the inhibitory actions of BoNT/C1 and BoNT/E, exhibiting a similar potency ratio to that observed in mouse lethality assays. It is concluded that NS-26 can serve as a good model for BoNT research.

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# CONTENTS

## SUMMARY

The botulinum neurotoxins (BoNTs), produced by strains of the anaerobic bacterium *Clostridium botulinum*, are the most poisonous substances known. These toxins cleave intracellular SNARE proteins within peripheral cholinergic neurons that participate in the docking and fusion of synaptic vesicles to transmitter release sites. BoNT-mediated cleavage of SNARE proteins results in inhibition of acetylcholine (ACh) release at the neuromuscular junction, leading to generalized muscle weakness, paralysis and death. Progress in our understanding of BoNT intoxication and in the development of therapeutic agents has been hindered by the lack of an adequate cellular model system where ACh release can be correlated with SNARE protein cleavage. To address this problem, we examined the NS-26 neuroblastoma cell line for suitability as a model for BoNT investigation. These cells are derived from a sympathetic ganglion tumor and have been reported to synthesize, store and release ACh and to form cholinergic synapses with skeletal muscle. In the current study, NS-26 cells were found to express the SNARE proteins SNAP-25 and syntaxin and to release ACh in a voltage- and $Ca^{2+}$-dependent fashion. ACh release was assessed by the uptake and release of radiolabeled ACh or by the quenching of the fluorescent dye FM1-43. For qualitative measure of synaptic activity, the latter was found to be as reliable as the radioisotope method but was more rapid and required less sample manipulation. Exposure of NS-26 cells to 10 nM BoNT/A resulted in complete inhibition of evoked ACh release accompanied by cleavage of ~35% of SNAP-25. NS-26 cells were also sensitive to the inhibitory actions of BoNT/C1 and BoNT/E, exhibiting a similar potency ratio to that observed in mouse lethality assays. It is concluded that NS-26 can serve as a good model for BoNT research.

## 1. INTRODUCTION

The botulinum neurotoxins (BoNTs) consist of seven immunologically distinct protein toxins (A-G) secreted by the anaerobic bacteria *Clostridium botulinum* (1-3). The BoNTs are the most lethal substance known to mankind, with an estimated human LD50 of 1-3 ng/kg (4, 5). Exposure to BoNT leads to symmetric descending flaccid paralysis due to inhibition of acetylcholine (ACh) release from motoneuron terminals, and death ensues from paralysis of the diaphragm and intercostal muscles (1). BoNTs belong to the family of A-B toxins where one component mediates binding (B) and the second contains the active (A) moiety. For BoNT, the binding component is the 100 kDa heavy chain (HC), and the active component is the 50 kDa light chain (LC); the latter acts by cleaving specific SNARE proteins in the nerve terminal cytoplasm (6). The SNARE complex consists of the synaptic vesicle protein synaptobrevin and two cytoplasmic proteins, SNAP-25 and syntaxin, both located at active zone specializations on the surface of the nerve terminal (7, 8). The interaction of synaptobrevin, syntaxin and SNAP-25 is responsible for the docking of the synaptic vesicle at release sites and for their subsequent fusion and release following depolarization-mediated increases in intracellular $Ca^{2+}$ (9).

In all reported cases of botulism, the toxin has been found to be highly selective for peripheral neuromuscular synapses (10). This selectivity stems from the targeting of peripheral cholinergic nerve terminals by the HC during the initial binding step (11, 12). The catalytic step that leads to inhibition of transmitter release is ubiquitous, since the same SNARE proteins are required for evoked release in all synapses (7, 8). In high concentrations, relatively insensitive systems such as CNS tissues, Aplysia ganglia and tumor cell lines can be effectively poisoned by clostridial neurotoxins (13-15).

Currently, there are no effective pharmacological treatments for botulism once symptoms appear (16). Progress in this area has been hindered by the lack of an adequate cellular model system for testing promising compounds. Primary dissociated murine spinal cord cells are potentially useful (17-20), but the population of cholinergic cells is difficult to maintain in culture (21).

The ideal model system for BoNT intoxication studies would be expected to meet the following criteria: 1) ability to form synapses with skeletal muscle, 2) release ACh in a voltage- and $Ca^{2+}$-dependent manner, 3) contain functional SNARE proteins and 4) display sensitivity to the intoxicating effects of BoNT. Previous studies have shown that NS-26 cells meet the first two requirements; criteria 2 - 4 were tested in the current study. Synaptic activity of NS-26 cells was monitored via release of $^{14}C$-labeled ACh and by release of the fluorescent styryl dye FM1-43 (23, 24). The results suggest that NS-26 cells are a good model for studies of BoNT intoxication and either release of $^{14}C$-labeled ACh or loss of FM1-43 staining can serve as an index of synaptic activity; the former is more precise, but the latter is more rapid.

## 2. MATERIALS AND METHODS

### *2.1 Cell Culture*

Stock NS-26 neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 $^{o}$C in a humidified atmosphere of 95% air/5% $CO_2$. To induce differentiation, which is required for optimal excitation-secretion coupling (25), cells were cultured in FBS-free DMEM containing N2 supplement (26) and 1 mM N-6,O-2'-dibutyryl adenosine cyclic 3':5'-monophosphate (DBcAMP) for 7-10 days. All experiments were performed on differentiated cells.

### *2.2 SNAP-25 Immunoprecipitation*

NS-26 cells were grown to 70% confluence in 25 $cm^2$ flasks and scraped into tubes containing 1.6 ml homogenization buffer consisting of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100 and 1X protease inhibitor cocktail. Cells were homogenized briefly with a hand held Tissue-Tearor™ (BioSpec Products, Inc., Bartlesville, OK). Homogenates were centrifuged at 100,000 x g for 1 hr at 4 °C. For immunoprecipitation studies, supernatants were transferred to 2 ml tubes and incubated at room temperature for 2 hr in the presence of SMI-81 anti-SNAP-25 mouse monoclonal antibody at a dilution of 1:500. The incubation was continued overnight at 4 °C after addition of 0.1 ml of a 30% suspension of Protein G-Sepharose. The immunocomplex was washed twice with homogenization buffer and once with distilled water. Immunoprecipitated proteins were eluted from the Protein G-Sepharose with 70 µl of 2X sample buffer (27), followed by incubation in a boiling water bath for 7 min.

### *2.3 SDS-PAGE and Immunoblotting*

Samples containing immunoprecipitated SNAP-25 were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) according to Laemmli (27). The Tris-HCl gel was composed of 13% separating gel, 5% stacking gel, and 3.3% cross-linking. The gels were run for 2 hr at 100 V in a solution containing 0.1 M Tris, 0.1 M Tricine and 0.1% (w/v) SDS at pH 8.3. For immunoblotting, proteins were transferred to polyvinylidene difluoride (PVDF) membranes for 1 hour at 100 V in transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 5% [v/v] methanol). Membranes were immediately transferred to blocking buffer consisting of Tris-buffered saline (TBS, 0.5 M NaCl, 20 mM Tris-HCl pH 7.5) and 3% bovine serum albumin (BSA), and incubated with gentle rocking for 2 hr at room temperature. PVDF membranes were incubated with SMI-81 or rabbit 2777 primary antibody (Table 1) in TTBS (TBS with 0.05% Tween 20) for 2 hr at room temperature with gentle agitation, followed by three sequential 10-minute washes in TTBS. Secondary antibody (Table 1) was applied in TTBS for 90 min at room temperature with gentle shaking. Blots were washed three times for 10 min in TTBS and once in TBS, and briefly rinsed with water prior to development with alkaline phosphatase 5-bromo-4-chloro-3-indoxyl-phosphate (BCIP)/nitroblue tetrazolium chloride (NBT) as substrate.

*2.4 Biotinylation of SMI-81*

SMI-81 (185 µl ) was incubated at room temperature with excess washed Protein G Sepharose in 20 mM sodium phosphate pH 7.0, and immunoglobins (Igs) were precipitated as described above (section 2.3). The Igs were eluted with 200 µl of 100 mM glycine-HCl (pH 3.0). The eluate was separated from the beads via centrifugation through a 0.45 µm spin column (Titan Bio-MSF Nylon), and neutralized with 0.1N NaOH. The neutralized Igs were buffer exchanged by gel filtration spin column. The FluoReporter Mini-Biotin-XX Protein Labeling Kit was used to biotinylate the SMI-81 antibody according to the manufacturer's directions.

*2.5 [$^{14}$C]-ACh Release Assay*

Differentiated NS-26 cells were incubated with or without BoNT LC for the indicated times. Toxins were removed by two washes with HEPES-buffered salt solution (HBS; 20 mM HEPES, pH 7.5, 150 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 25 mM glucose) at room temperature. Cells were then incubated in media containing 40 µM [$^{14}$C]-choline chloride for 3 hr at 37 °C. Extracellular radioactivity was removed by two sequential 1-minute washes with HBS at room temperature and one 15-minute HBS wash at 37 °C. Cells were then incubated for 15 minutes at 37 °C in either HBS (non-stimulated control) or in HBS containing 50 mM KCl (HBS-HK) to stimulate transmitter release. HBS and HBS-HK solutions were collected and centrifuged at 16,000 x g for 3 min to remove any cell debris, and radioactivity in the supernatant was determined. Cells were dissolved in 1% SDS and incubated in a boiling water bath for 10 min, and the radioactivity remaining in the cells was quantified. Protein in the cell suspension was determined by the Bradford assay (28). [$^{14}$C]-ACh release was expressed as dpm/mg protein. The remaining sample of the cell suspension was used for determining SNAP-25 cleavage.

*2.6 Uptake and Release of FM1-43*

Uptake and release of the fluorescent dye FM1-43 were initiated by depolarization in high $K^+$ (80 mM KCl) as described (29). For dye loading, cultures grown on CELLocate 175 µm coverslips were depolarized with HBS-HK containing 10 µM FM1-43 for 5 min at 37 $^{o}$C and rinsed 3 times to remove excess dye. Release of FM1-43 was initiated by a 10-minute stimulation with dye-free HBS-HK. Depolarization in the absence of dye leads to de-staining of cells, which is a measure of transmitter efflux. To test the $Ca^{2+}$ dependence of transmitter uptake and release, $Ca^{2+}$ was replaced with $Mg^{2+}$, and 0.5 mM ethylene glycol bis(β-aminoethylether)- N,N,N',N'-tetraacetic acid (EGTA) was added to chelate trace levels of $Ca^{2+}$.

Fluorescent images of cells were obtained with a Zeiss Axioskop epifluorescence microscope equipped with a 40x Achroplan water-immersion objective (0.75 NA), a 100-W Hg lamp, 5-100% neutral density transmission filters, excitation filters (435/10 nm), dichroic mirrors (455 nm) and emission filters (540/100 nm). Images were captured with AxioVision image software and stored.

*2.7 Materials*

NS-26 neuroblastoma cells (passage 14) were kindly provided by Dr. Marshall W. Nirenberg (NIH, Bethesda, MD). DMEM, FBS, DBcAMP and all routine reagents were purchased from Sigma-Aldrich (St. Louis, MO). CELLocate coverslips were obtained from Eppendorf, Inc. (Westbury, NY). Pure BoNT LCs were provided by Matabiologics (Madison, WI). $^{3}$[H]-choline (specific activity 55 mCi/mmol) was from ICN (Costa Mesa, CA). FM1-43 and FluoReporter Mini-Biotin-XX Protein labeling kit were from Molecular Probes, Inc. (Eugene, OR). Primary and secondary antibodies were obtained from the indicated sources (Table 1).

**TABLE 1**

**Antibodies for Immunoblotting**

| Name | Antibody To | Source / Type | Final Dilution |
|---|---|---|---|
| SMI-81 | mammalian SNAP-25 | Sternberger Monoclonals, Lutherville, MD monoclonal mouse ascites | 1:2,000 |
| Rabbit 2777 | mouse SNAP-25 NLRHMALDMGNEIDTQNRQID | BioSynthesis, Inc. Lewisville, TX polyclonal rabbit serum | 1:1,000 |
| HPC-1 | Mouse syntaxin | Abcam Cambridge, UK monoclonal | 1:500 |
| anti-Mouse | mouse IgG | Sigma-Aldrich # A-1682 St. Louis, MO goat, alkaline phosphatase conjugate | 1:10,000 |
| anti-Biotin | biotin | Sigma-Aldrich # A-7064 St. Louis, MO goat, alkaline phosphatase conjugate | 1:30,000 |

## 3. RESULTS

### *3.1 Differentiation of NS-26 Cells*

Clonal NS-26 cells grown in DMEM and 5% FBS undergo rapid division (doubling time ~22 hr) and provide a large population of uniform cells for BoNT studies (Fig. 1A). In this state of rapid division, however, the cells exhibit poor electrical excitability, low rates of synapse formation, general absence of neurites and little or no depolarization-dependent release of ACh (22). Substituting the nutrient mixture N2 (26) for FBS led to a marked slowing of cell proliferation and to a more stable population (Fig. 1B). Addition of 1 mM DBcAMP to the N2-supplemented growth medium caused a further decrease in cell division and the appearance of highly differentiated cells. DBcAMP-treated cells were characterized by multipolar shapes and long, highly branched dendritic processes (Fig. 1C). The action of DBcAMP required at least 4 days and was optimal after 7-10 days, as described by Adler et al. (22). Differentiated cells could be maintained in DBcAMP-containing media for at least 6 weeks (22). All experiments reported here were performed on cells differentiated for 7-10 days.

### *3.2 NS-26 Cells Contain Syntaxin and SNAP-25*

The lack of adequate cellular model systems has hindered progress in the development of effective pharmacological treatment for BoNT intoxication due to the difficulties of large scale testing of drugs in isolated muscles or whole animals (30). To determine whether NS-26 cells would be suitable for BoNT studies, it was necessary to 1) demonstrate the presence of SNAP-25, 2) show that isolated SNAP-25 from NS-26 cells is cleaved by BoNT and 3) determine whether intact NS-26 cells are sensitive to BoNT-mediated inhibition of ACh release.

The presence of SNARE proteins in NS-26 cells was demonstrated by immunocytochemical methods as shown in Fig. 2. Differentiated NS-26 cells were incubated with SMI-81 anti-SNAP-25 antibody at a dilution of 1:2000 or with HPC-1 anti-syntaxin antibody at 1:500 followed by incubation with anti-mouse IgG (H-L)-AF488 secondary antibody. Diffuse staining for SNAP-25 (Fig. 2A) or syntaxin (Fig. 2B) was observed in addition to a more intense band of staining associated with the inner plasma membrane of cell bodies and processes. The latter is expected since both SNAP-25 and syntaxin are highly localized on the inner surface of the plasma membrane where they participate in vesicle docking, fusion and release (31).

### *3.3 BoNT-Mediated Cleavage of SNAP-25*

Although immunocytochemical staining was able to disclose the presence of SNARE proteins in NS-26 cells, it could not be used to demonstrate BoNT-mediated cleavage of these proteins since the antibodies SMI-81 and HPC-1 do not discriminate between intact and cleaved SNARE proteins. Since cleavage of syntaxin is not involved in human BoNT intoxications (10), the remaining efforts were concentrated exclusively on SNAP-25. To demonstrate that SNAP-25 from NS-26 cells is susceptible to cleavage by BoNT, cells were homogenized in Triton-X-100, incubated in the presence of 28 nM BoNT/A, 72 nM BoNT/E or 720 nM BoNT/C1 LCs for 4 hr at 37 $^{o}$C and subjected to SDS-PAGE and immunoblotting (Fig. 3). In the absence of toxin, SNAP-25 appears as a single band

of 25 kDa, as has been reported for SNAP-25 isolated from mouse and human brain (32). Incubation of SNAP-25 with BoNT/A or BoNT/E LC resulted in almost complete cleavage of SNAP-25 and the appearance of the expected truncated SNAP-25: a 24 kDA fragment from loss of the C-terminal 9 residues following action of BoNT/A or a 22 kDA fragment from loss of the C-terminal 26 residues following action of BoNT/E (33). BoNT/C1 was considerably less potent, and 720 nM only cleaved approximately 20% of the SNAP-25. This is consistent with the low potency of BoNT/C1 for SNAP-25 cleavage (34).

*3.4 ACh Release Is Inhibited by BoNT*

Having demonstrated that SNAP-25 isolated from NS-26 homogenates is cleaved by BoNT, it is necessary to show that SNAP-25 is also cleaved when intact cells are exposed to toxin. Furthermore, it must also be established that cleavage of SNAP-25 in NS-26 cells leads to inhibition of ACh release. Thus, cells were incubated in [$^{14}$C]-choline and depolarized with high $K^+$ to evoke the release of [$^{14}$C]-ACh. At the conclusion of the release experiments, cells were lysed, and cleavage of SNAP-25 was determined so that BoNT-mediated alterations of ACh release could be compared with the degree of cleavage of SNAP-25 in the same cultures.

Fig. 4 shows that treatment with 10 nM BoNT/A resulted in total inhibition of [$^{14}$C]-ACh release and concomitant cleavage of ~35% of the cellular SNAP-25 (Fig. 4, inset). Although the NS-26 cells were less sensitive to BoNT/E, this serotype also inhibited [$^{14}$C]-ACh release at concentrations that produced a corresponding cleavage of SNAP-25. These data are in agreement with that of Keller *et al.* (35), who reported that cleavage of SNAP-25 by BoNT results in a greater than proportional inhibition of transmitter release. Since SNAP-25 cleavage and inhibition of ACh release were observed in intact cells, it can be deduced that NS-26 cells possess the required toxin receptors and mechanism to internalize toxin and deliver the LC to the SNARE protein for cleavage (36).

*3.5 NS-26 Cells Take Up and Release FM1-43*

Although the radioisotope method for ACh release is reliable, it also is time consuming, is labor intensive and generates radioactive waste (37). To determine the feasibility of studying synaptic activity using a non-radioactive method in NS-26 cells, the styryl dye FM1-43 was used to examine synaptic vesicle release. Incubation of NS-26 cells in 80 mM $K^+$ buffer containing 10 μM FM1-43 for 5 minutes at 37 °C was sufficient to load cells with dye (Fig. 5). When imaged for FM1-43 fluorescence, numerous discreet spots (puncta) were observed at the periphery of cells and in processes. These puncta represent clusters of synaptic vesicles that take up dye during vesicle recycling (29). Release of dye was accomplished with a 10-minute incubation in 80 mM $K^+$ buffer lacking FM1-43 (Fig. 5B). Essentially all FM-143 fluorescence was released under these conditions, suggesting that FM1-43 is a reliable marker of synaptic activity. No morphological abnormalities were observed after FM1-43 loading (Fig. 5, C-D).

To determine if the FM1-43 was released in a $Ca^{2+}$-dependent manner, as would be expected for regulated exocytosis, $Ca^{2+}$ was replaced in all buffers with an equimolar concentration of $Mg^{2+}$, and EGTA was added to remove any traces of $Ca^{2+}$. In $Ca^{2+}$-free buffer, NS-26 cells took up FM1-43 (Fig. 6A); however, the dye was not released upon a

second depolarizing stimulus with 80 mM $K^+$ in FM1-43-free solution (Fig. 5B). These results indicate that release of FM1-43 exhibits the expected $Ca^{2+}$-dependence for regulated exocytosis.

*3.6 BoNT/A Inhibits FM1-43 Release, but Not Uptake in NS-26 Cells*

The finding that FM1-43 release in NS26 cells was depolarization- and $Ca^{2+}$-dependent suggests that FM1-43 fluorescence may be a good candidate for replacement of the radioisotope technique in studying the action of BoNT. To demonstrate the use of FM1-43 as a marker of BoNT action, NS-26 cells were treated with 20 nM BoNT/A in growth medium for 24 hours at 37 °C to inhibit ACh release. At the end of this period, cells were washed and incubated with 80 mM FM1-43 for 5 min to initiate dye uptake. BoNT/A-treated NS-26 cells were able to take up FM1-43 (Fig. 7A) but were unable to release the dye following a 10-minute stimulation by 80 mM $K^+$ (Fig. 7B). These data suggest that BoNT/A blocks the exocytic pathway needed to release FM1-43, and that FM1-43 is an appropriate monitor of BoNT-mediated inhibition of transmitter release.

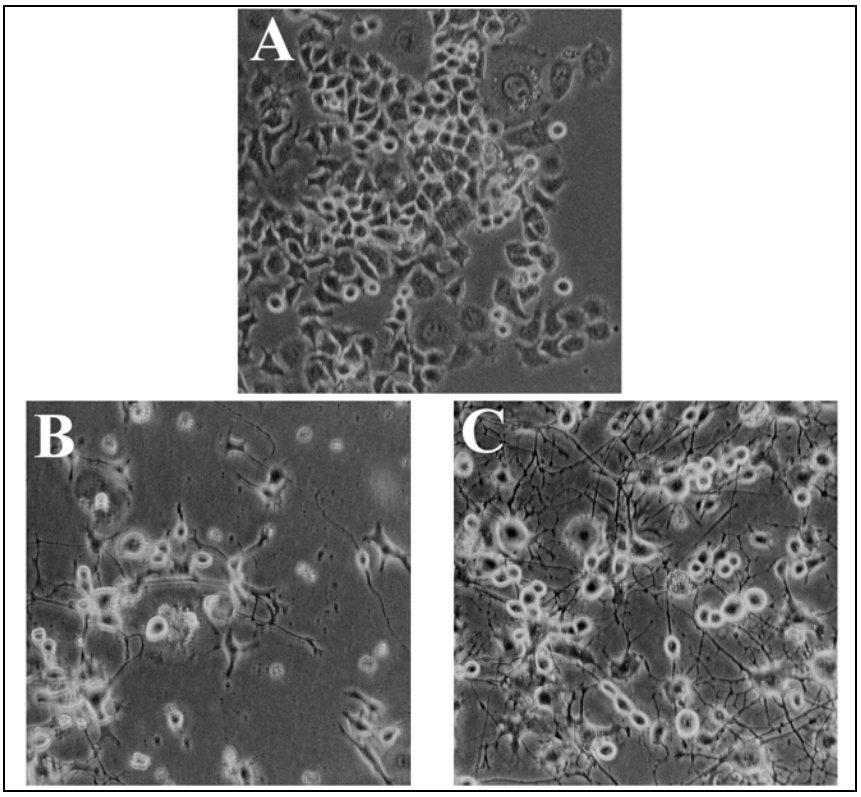


**Figure 1.** Phase contrast photomicrographs of NS-26 cells grown in DMEM supplemented with 5% FBS (A), N2 nutrient mixture (B) or N2 plus 1 mM DBcAMP. Cells were seeded in 12-well tissue culture plates at a density of $10^5$ per well and grown under the indicated conditions for 7-9 days.

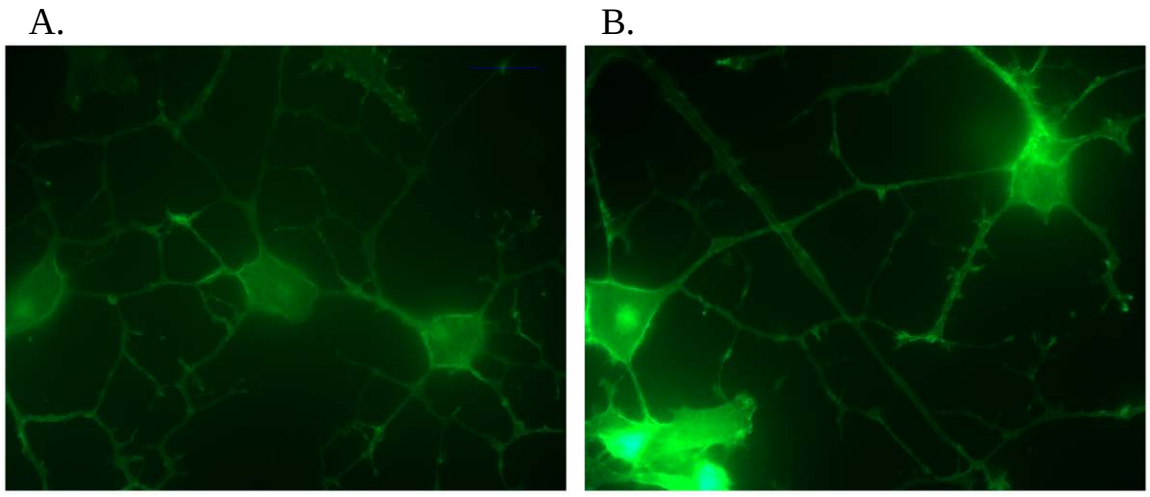


**Figure 2.** Differentiated NS-26 cells were immunostained for (A) SNAP-25 or (B) syntaxin. After washing, fixation and blocking with 10% normal goat serum in PBS, antibodies were applied in PBS/1% BSA for 1 hr using SMI-81 antibody at 1:2000 for SNAP-25 and HPC-1 mAb at 1:500 for syntaxin. Incubation in primary antibody was followed by staining with anti-mouse IgG (H + L)-AF488 secondary antibody at 1:200. Cells were visualized with fluorescence microscopy.

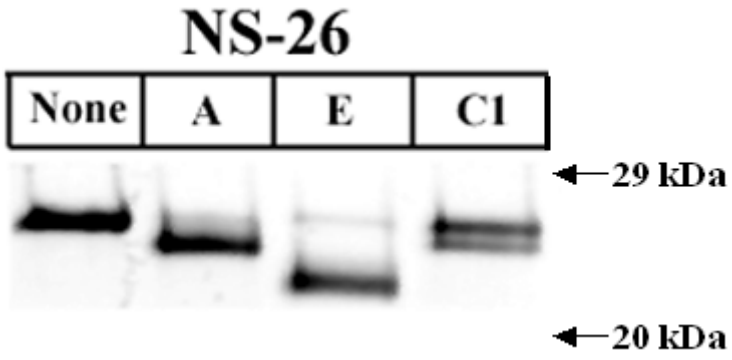


**Figure 3.** In vitro cleavage of SNAP-25 by 28 nM BoNT/A, 72 nM BoNT/E or 720 nM BoNT/C1 LCs. The neurotoxin LCs were added to extracts of NS-26 cells for 4 hr at 37 $^{o}$C. Full-length and truncated SNAP-25 were immunoprecipitated with SMI-81. Detection of SNAP-25 was achieved with polyclonal rabbit antiserum 2777.

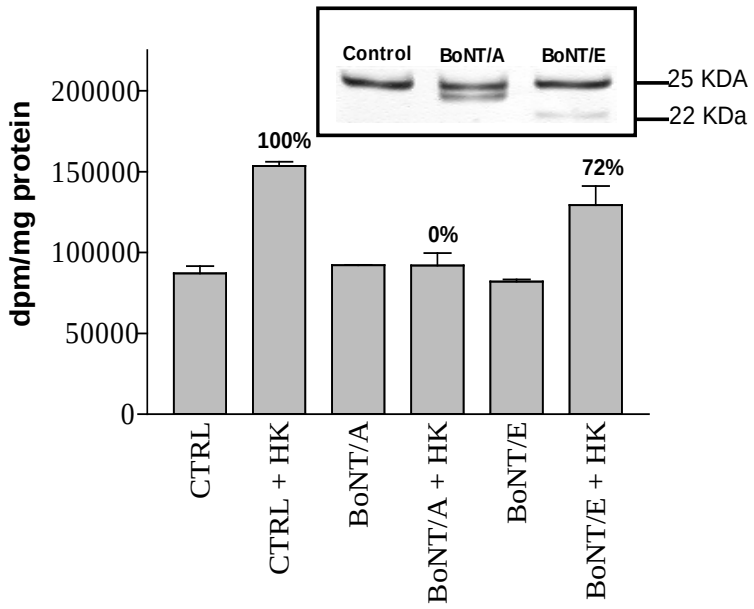


**Figure 4.** [$^{14}$C]-ACh release from NS-26 cells is inhibited by BoNT. NS-26 cells were exposed to 10 nM BoNT/A or 25 nM BoNT/E for 2.5 days, washed free of extracellular toxin and assayed for release. Basal efflux of radioactivity was constant for the three non-depolarizing conditions: control (CTRL), BoNT/A and BoNT/E. High $K^+$-induced release of [$^{14}$C]-ACh (HK) was measured after 15 min of stimulation with 80 mM KCl. The inset shows SNAP-25 immunoblots (biotinylated SMI-81 as the antibody) of one sample from each treatment group at the end of the high $K^+$ stimulation. The numbers above the HK histograms represent depolarization-evoked [$^{14}$C]-ACh release as a % of that observed in the absence of toxin.

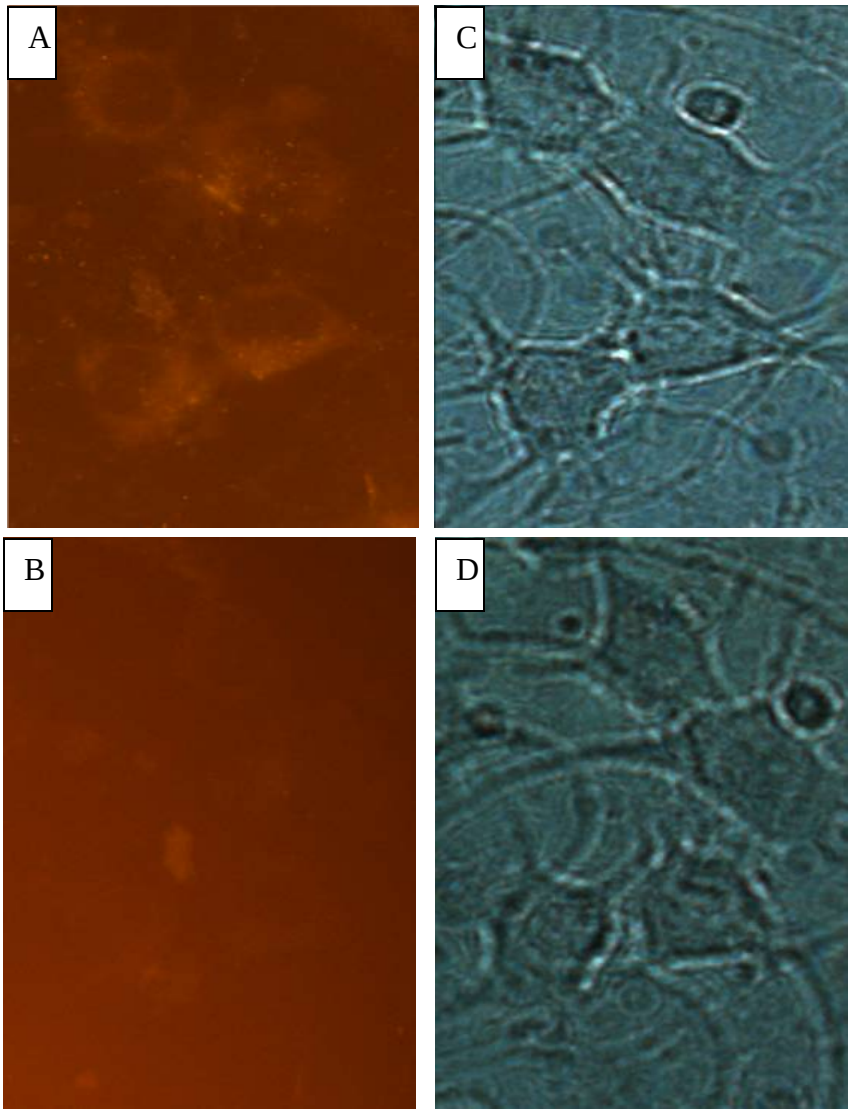


**Figure 5.** NS-26 cells take up and release the styryl dye FM1-43. Endocytotic uptake of FM1-43 was elicited by stimulation of cells with high $K^+$ for 5 min in the presence of 10 μM FM1-43 (A). After cells were washed to remove extracellular dye, a second depolarization with 80 mM $K^+$ led to release of FM 1-43 as indicated by a quenching of fluorescence (B). Panels (C) and (D) are brightfield images that correspond to the fields in (A) and (B) respectively.

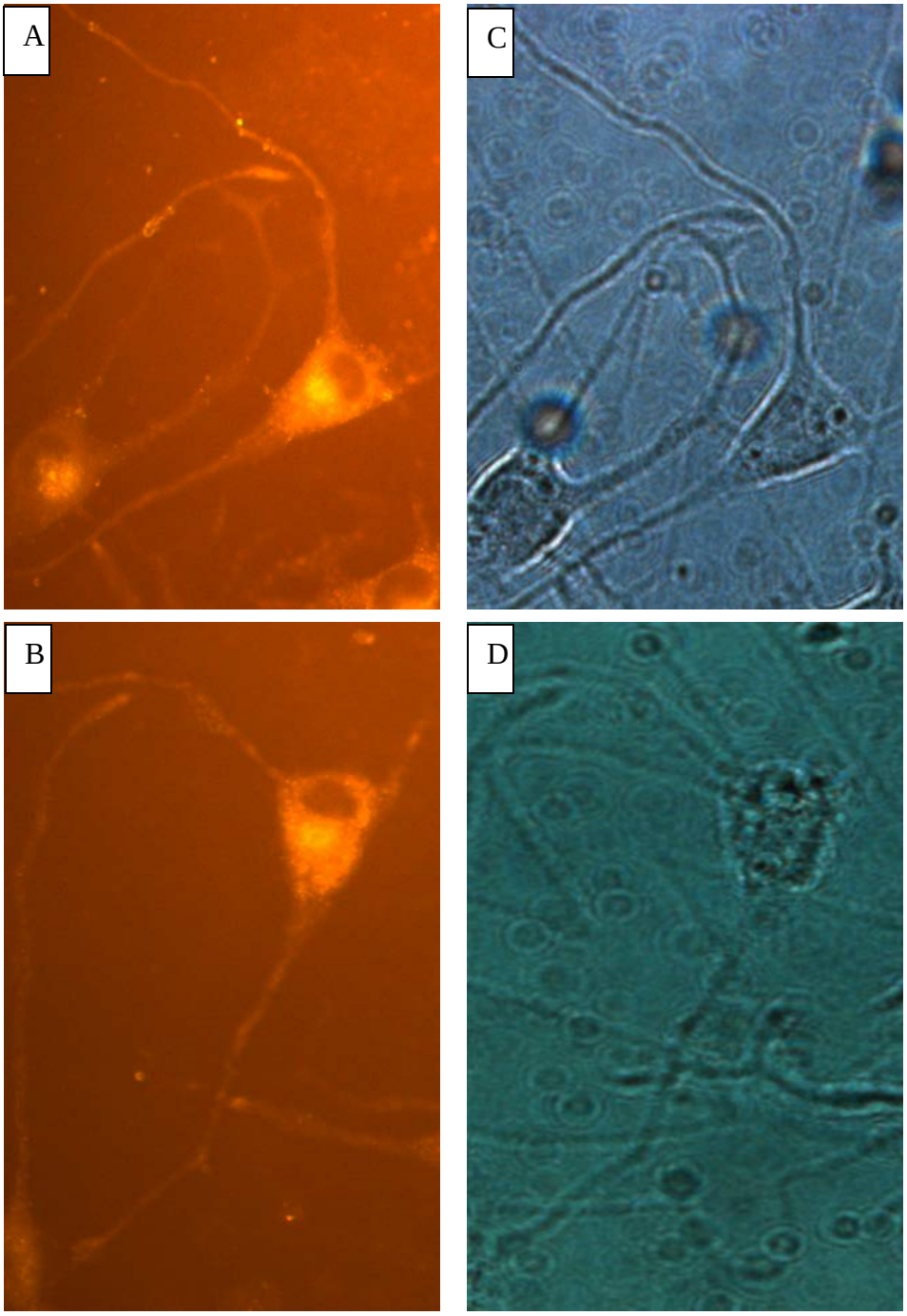


**Figure 6.** Release of FM 1-43 is $Ca^{2+}$-dependent. Cells were incubated with 10 μM FM1-43 in 80 mM $K^+$ (A), washed to remove excess dye and incubated for 10 min in 80 mM $K^+$ to elicit release (B). $Ca^{2+}$ was omitted from all solutions, and 1 mM EGTA was added to chelate trace levels of $Ca^{2+}$. In the absence of $Ca^{2+}$, cells take up but do not release FM1-43. Panels (C) and (D) are brightfield images that correspond to the fields in (A) and (B) respectively.

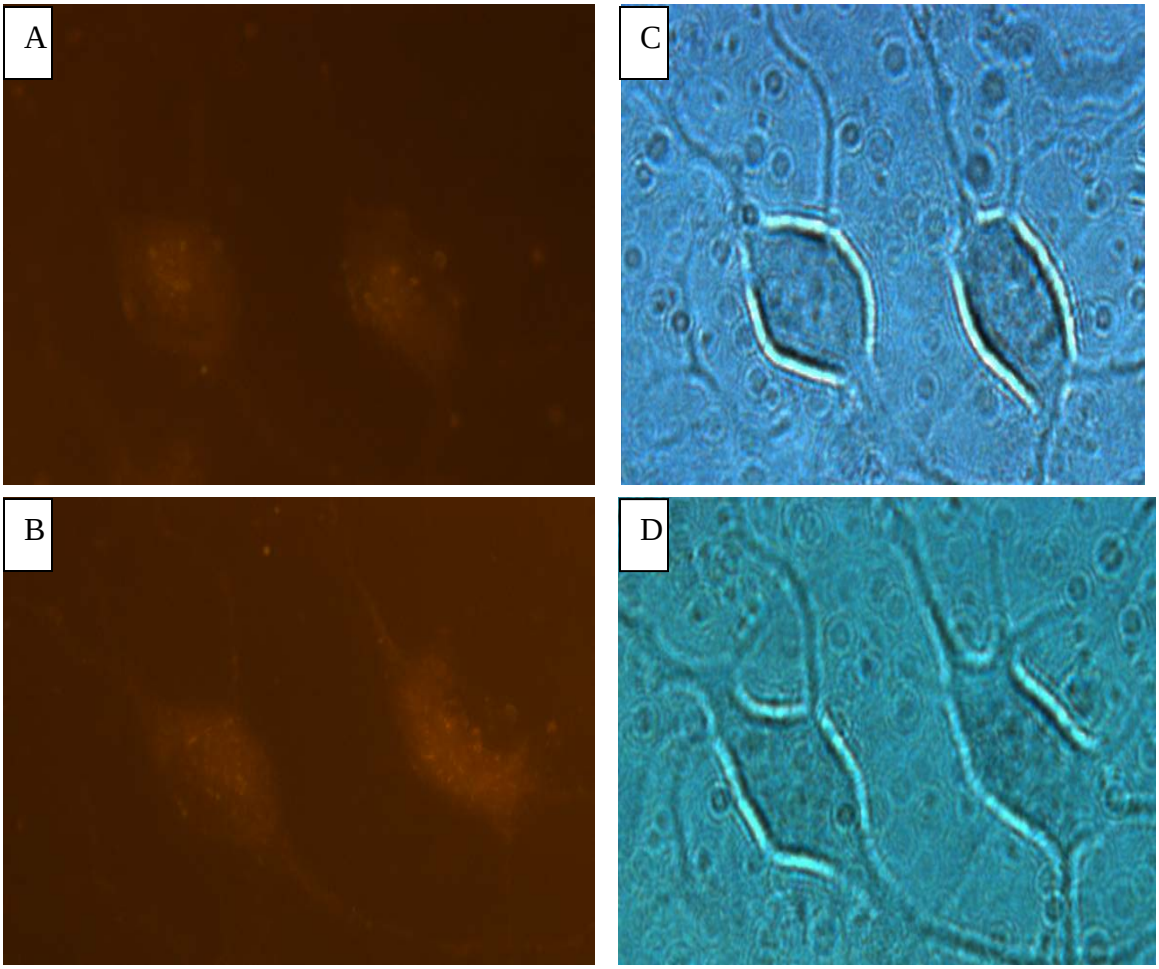


**Figure 7.** Release of FM1-43 is inhibited by 20 nM BoNT/A. Cells were exposed to 20 nM BoNT/A neurotoxin for 24 hr and incubated with 10 μM FM1-43 in 80 mM $K^+$ (A). After washing to remove excess dye, a 10 min depolarization with 80 mM $K^+$ failed to elicit release of FM1-43 (B). Panels (C) and (D) are brightfield images that correspond to the fields in (A) and (B) respectively.

## 4. DISCUSSION

Development of post-exposure pharmacological treatments for BoNT intoxication is of paramount importance. Successful drug development requires a detailed understanding of the basis for persistence of BoNT intoxication as well as for the binding, internalization, intracellular trafficking and mechanism of toxin-mediated cleavage of the SNARE proteins. A simple and readily available cell culture system is desirable for studies on the mechanism of action of BoNT and for rapid screening of potential therapeutic agents for efficacy and toxicity. Traditionally, whole animals, isolated nerve muscle preparations and primary dissociated murine spinal cord cells have been used as model systems for BoNT intoxication (10). The animal and isolated tissue models have been found to be too slow and costly for rapid screening (38). Primary spinal cord cultures are highly sensitive to BoNT. There are, however, several disadvantages of using these cultures: they require embryonic tissue for each plating, the neuronal cell population yield is relatively low and the relevant cholinergic motor neurons are not stable in culture (20, 21). In principle, a continuous neuronal cell line could overcome these difficulties and allow for simultaneous assessment of inhibition of transmitter release and cleavage of SNARE proteins associated with BoNT intoxication. Initial studies with the well-characterized NG108-15 neuroblastoma-glioma hybrid cells indicated that this line was unsuitable for BoNT research due to its low adherence to substrates generally used in monolayer cell culture. Routine manipulations such as drug additions or solution changes resulted in substantial loss of cells. The cholinergic NS-26 neuroblastoma cell line has been reported to adhere strongly to surfaces used in cell culture (22) and therefore appeared to be a better candidate for a cellular model of BoNT intoxication.

When appropriately differentiated, NS-26 cells are able to form synapses with skeletal muscle and to release ACh in a voltage- and $Ca^{2+}$-dependent fashion (22). In the present study, two other criteria were tested: the presence of the SNARE proteins SNAP-25 and syntaxin and susceptibility of the cells to BoNT intoxication. The presence of SNARE proteins was established by immunocytochemical techniques (Fig. 2) and Western blot analysis (Fig. 3). Sensitivity of NS-26 cells to BoNT intoxication was demonstrated by inhibition of evoked release of [$^{14}$C]-ACh following exposure to BoNT/A or BoNT/E. Inhibition of release correlated with the SNAP-25 cleavage, as reported by Keller *et al.*(35). Cleavage of 35% of SNAP-25 by BoNT/A resulted in a complete block of release. In addition to establishing a neuronal cell line for BoNT intoxication, development of a non-radioactive assay for synaptic activity was also desired. Styryl dyes reversibly stain membranes, but are unable to penetrate membranes, providing a fluorescent tool to specifically study endo- and exocytosis. The styryl dye FM1-43 has been used extensively to study vesicle cycling in neuronal preparations. Cochilla *et al.* (39) and Henkel *et al.* (40) have shown that FM1-43 is incorporated into synaptic vesicles. FM1-43 has also been used for quantal analysis of neurotransmitter release (41). The staining pattern of FM1-43 is usually punctate, indicating the location of the endocytosed vesicles (39). Due to the wide use of FM1-43 to study vesicle cycling in neurons, this dye was chosen as a non-radioactive alternative to studying synaptic activity in NS-26 cells.

The NS-26 cells were able to take up and release the FM1-43 dye (Fig. 5). Localized fluorescence was observed near the cell nucleus and may indicate staining of the Golgi and endoplasmic reticulum in addition to synaptic vesicles (Fig. 5). Staining of other organelles by styryl dyes has been reported (39). The release of FM1-43 from NS-26 cells was $Ca^{2+}$-dependent (Fig. 6B), suggesting that release is mediated by the process of regulated exocytosis. Since endocytosis is not dependent upon external $Ca^{2+}$ (42, 43), NS-26 cells were able to take up FM1-43 in the absence of $Ca^{2+}$ (Fig. 6A). NS-26 cells treated with BoNT/A also took up FM1-43, but could not release the dye upon high $K^{+}$ stimulation (Fig. 7). This suggests that BoNT/A-treated NS-26 cells retain a functional endocytosis machinery, but have an impaired functional exocytosis pathway for release of dye.

When compared to highly active tissues such as the neuromuscular junction, the fluorescence intensity of FM1-43 was low, containing both specific punctate staining as well as non-specific diffuse staining (Figs. 5-7). As a result, FM1-43 can best be used as a qualitative measure of inhibition of transmitter release; more precise and quantitative determinations will still require the radioisotope release assay. The low overall fluorescence intensity of FM1-43 in NS-26 cells may be due to their low endocytic and/or exocytic activity. In future studies, growth factors for enhancing synaptic activity will be explored to increase fluorescence intensity and reduce the non-specific staining pattern of the FM1-43 dye.

## 5. CONCLUSIONS

The NS-26 cell line meets several criteria that make it useful as a model cholinergic system to study BoNT intoxication: 1) ability to form synapses with muscle; 2) ability to release ACh; 3) presence of the SNARE proteins; and 4) sensitivity to BoNT. The NS-26 cells provide a new tool to examine the turnover rates of BoNT cleavage products and trafficking patterns of the BoNT LCs. In addition, these cells will allow efficacy and toxicity studies for potential BoNT therapeutic agents.

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