ABSTRACT

JOSHUA J. SUMISLAWSKI
Botulinum Neurotoxin Serotype A Promotes the Neurite Outgrowth of Embryonic Motor
Neurons via cAMP-Dependent Intracellular Signaling
(Under the Direction of JULIE A. COFFIELD)

Botulinum neurotoxin, the agent that causes the paralytic disease botulism, is the most
poisonous substance known. The ability of botulinum neurotoxin serotype A (BoNT/A) to
produce flaccid paralysis makes it both of public-health concern as a biological weapon and of
medical interest as a versatile therapeutic agent (BOTOX®). Less appreciated is the frequent
observation that poisoned nerve endings respond by initiating and extending neurites. This
phenomenon, termed "sprouting," has now been proposed as an indication that the binding of
BoNT/A to the neuronal membrane activates intracellular signals that are independent of its
already well-characterized paralytic action. Supporting this novel idea, recent studies have
demonstrated that the ability of the toxin to induce sprouting is concentration-dependent and
counteracted by receptor antagonists. This study addressed the hypothesis that BoNT/A
promotes neurite outgrowth through a signaling pathway that depends on the second messenger
cyclic adenosine monophosphate (cAMP). To test this hypothesis, primary cultures of motor
neurons from embryonic mice were exposed to BoNT/A with and without several inhibitors of
cAMP-dependent pathways. In the presence of the inhibitors, the ability of the toxin to induce
sprouting was eliminated. These results suggest that BoNT/A promotes neurite outgrowth of
embryonic motor neurons via cAMP-dependent intracellular signaling. Additional studies are
necessary to further our understanding of the neurotrophic action of this potent toxin.

INDEX WORDS: Botulinum neurotoxin, Neurite outgrowth, Embryonic motor neurons,
                            cAMP

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OF EMBRYONIC MOTOR NEURONS VIA cAMP-DEPENDENT INTRACELLULAR

SIGNALING

by

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Botulinum neurotoxin is the causative agent of botulism

Derived from the Latin word *botulus*, meaning "sausage," botulism is a paralytic and potentially fatal disease that was first associated with the consumption of spoiled sausage in Europe during the eighteenth century. The offending agent in the sausage was suspected to be prussic acid, now called hydrocyanic acid, until the German physician and poet Justinus Kerner systematically studied and described the disease in 1817. Although he was unable to identify its source, Kerner determined that a biological agent, not inorganic, which develops under anaerobic conditions in the sausage is responsible for the clinical symptoms of botulism[1,2]. Kerner's hypothesis was confirmed when the Belgian microbiologist Emile Pierre van Ermengem isolated the anaerobic bacterium *Clostridium botulinum* (Figure 1.1) from a ham implicated in an outbreak of the disease in 1895[3]. Recognizing its potential use as a biological weapon, the United States Army began research on botulism, which resulted in the purification of botulinum neurotoxin (BoNT) by the end of World War II[4].
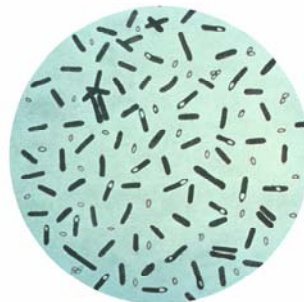

Figure 1.1: Photomicrograph of *C. botulinum*[5]. *C. botulinum* is a rod-shaped, gram-positive, anaerobic bacterium that is found ubiquitously in soil and produces botulinum neurotoxin.

Seven immunologically distinct serotypes of BoNT, denoted A-G, have since been isolated from three species of *Clostridium*: *C. botulinum*, *C. baratii*, and *C. butryicum*[1]. When they are obtained in a purified form, all seven serotypes exist as polypeptides composed of a 50-kDa light chain linked by a disulfide bond to a 100-kDa heavy chain (Figure 1.2). These toxins induce the paralytic effects of botulism by potently inhibiting neurotransmission, but only BoNT/A, B, E, and F cause the disease in humans[6]. Because it is lethal in very small doses and could easily be distributed throughout large populations, BoNT is one of the Centers for Disease Control and Prevention's six Category A bioterrorism agents. Despite its extreme potency, it is ironically used as a versatile therapeutic agent (BOTOX®) to treat a variety of neuromuscular disorders and cosmetic conditions.
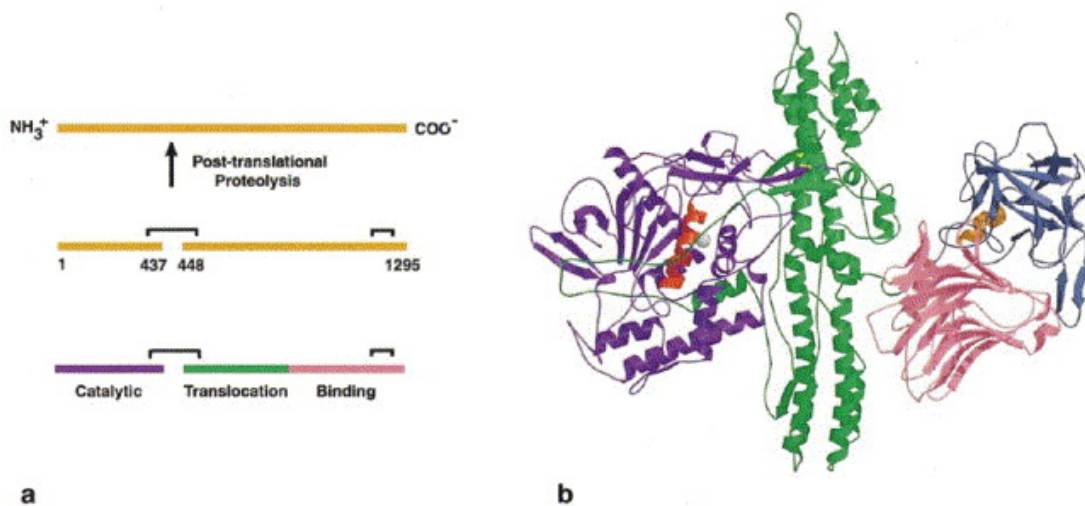
Figure 1.2: Quaternary structure of BoNT/A[7]**.** (a) The toxin is synthesized as an inactive 150-kDa single-chain peptide, which is subsequently cleaved into a 50-kDa light chain and a 100-kDa heavy chain. One of two disulfide bonds links these chains. The catalytic domain (purple) constitutes the light chain, and the translocation domain (green) and the binding domain (pink) constitute the heavy chain. (b) Backbone trace.

BoNT produces flaccid paralysis by cleaving SNARE proteins

The most understood action of BoNT is its ability to prevent muscles from contracting (Figure 1.3). To induce this effect, BoNT potently inhibits neurotransmission at the neuromuscular junction via a four-step mechanism[2]. The binding of the C-terminal domain of the heavy chain to the neuronal membrane (step 1) triggers endocytosis of the toxin into clathrin-coated vesicles (step 2), which are acidified by proton pumps. At the low pH of the vesicular lumen, the N-terminal domain of the heavy chain integrates into the vesicular membrane, and the light chain translocates into the cytoplasm (step 3), where it cleaves specific sites on SNARE proteins (step 4). BoNT/B, D, F, and G target synaptobrevin; A, C, and E target SNAP-25; C targets syntaxin. As a result of SNARE proteolysis, acetylcholine, the neurotransmitter that normally stimulates muscles to contract, cannot be released by the motor neuron (Figure 1.4). Exposure to this toxin can ultimately become fatal in animals and humans when it inhibits neurotransmission from the phrenic nerve to the thoracic diaphragm, leading to respiratory failure which results in death. SNARE proteolysis by the light chain is today widely considered the primary intracellular action of BoNT.

Figure 1.3: Six-week-old infant with botulism[8]. BoNT produces flaccid paralysis, which is apparent in the loss of muscle tone in this infant with botulism, especially in the head and neck.
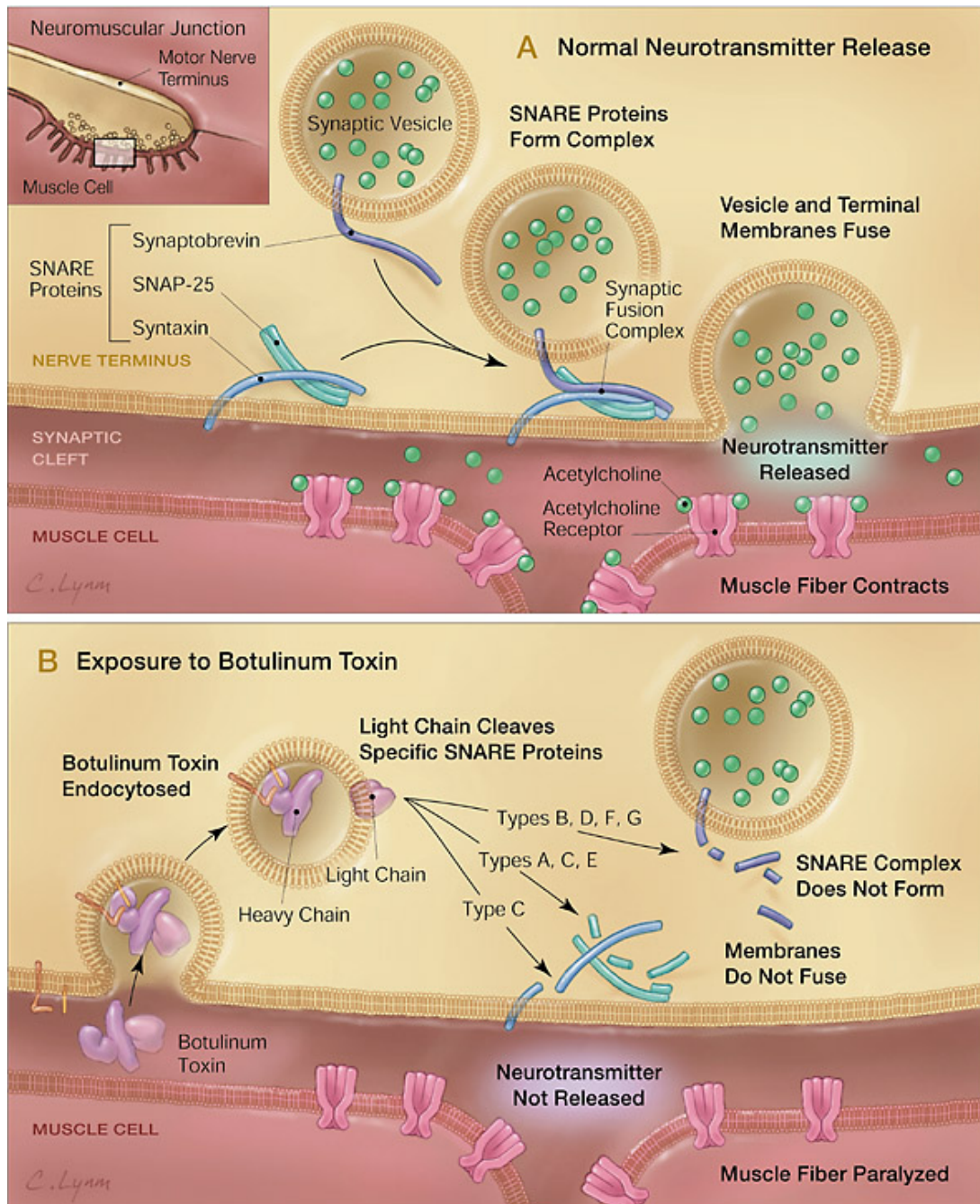
Figure 1.4: Mechanism and target sites of the paralytic action of BoNT[9]. (a) During normal neurotransmitter release at the neuromuscular junction, SNARE proteins form a complex of coiled coils that allows the membrane of the synaptic vesicle containing acetylcholine to fuse with the neuronal membrane. Acetylcholine is then released into the synaptic cleft and binds its receptor on the muscle cell. (b) After BoNT enters the neuron via receptor-mediated endocytosis, the light chain specifically cleaves the SNARE proteins. BoNT/B, D, F, and G target synaptobrevin; A, C, and E target SNAP-25; C targets syntaxin. Without the SNARE proteins to mediate acetylcholine release into the synaptic cleft, the muscle cannot contract.

BoNT/A promotes the outgrowth of embryonic motor neurons

In studies of the proteolytic action of BoNT, it was frequently observed that poisoned nerve endings respond by initiating and extending neurites, a phenomenon that has since been termed "sprouting" (Figure 1.5). This outgrowth helps neurons to regain their function in neurotransmission after the toxin cleaves SNARE proteins[10]. However, because BoNT-induced sprouting has widely been assumed to be a nonspecific secondary response to denervation, the direct molecular mechanisms that mediate this outgrowth at the terminal bouton are still poorly understood. Furthermore, sprouting has only been reported thus far in neurons poisoned with BoNT/A, D, and F.
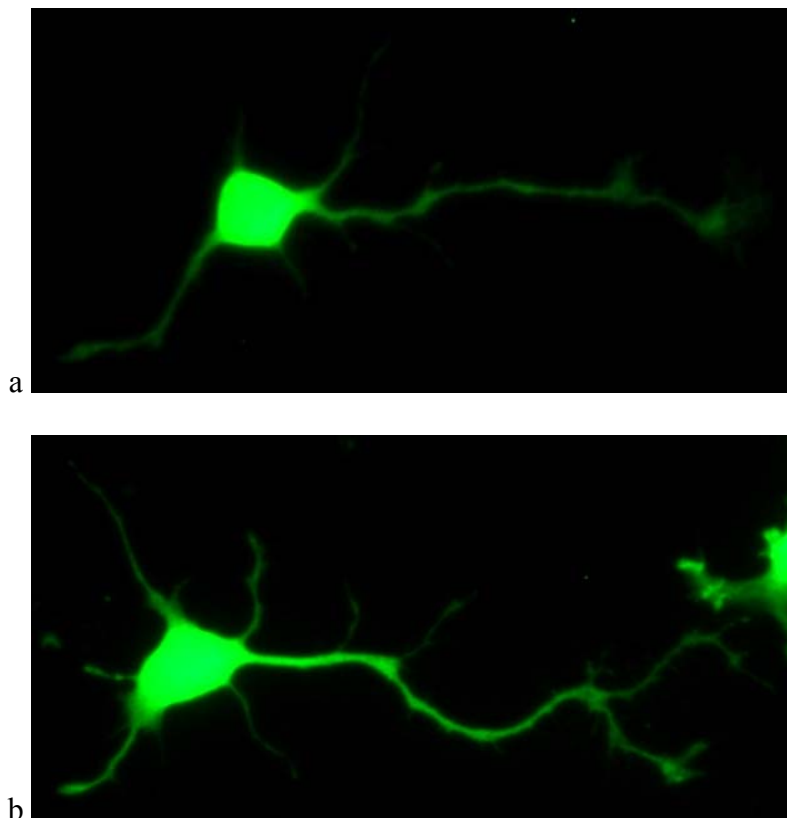

Figure 1.5: BoNT/A-induced sprouting. These fluorescent motor neurons were cultured without (a) and with (b) BoNT/A. Compared with the control, the poisoned neuron exhibits increased and extended neurites.

In the first study to address other possible intracellular actions that might account for BoNT-induced sprouting[11], the effects of BoNT/A were examined on embryonic motor neurons (eMNs) cultured from mouse spinal cords. BoNT/A stimulated neurite outgrowth in a concentration-dependent manner. This effect was mimicked by the heavy chain, which was isolated from the holotoxin (Figure 1.2); therefore, sprouting occurs independently of the proteolytic action, which is mediated solely by the light chain. Further supporting this idea, the ability of the toxin to induce sprouting was eliminated in the presence of *Triticum vulgaris* lectin, an antagonist of the binding of all seven serotypes of BoNT. Outgrowth was also stimulated regardless of SNAP-25 cleavage. These results collectively suggest that BoNT/A promotes the neurite outgrowth of eMNs through a receptor-mediated intracellular signaling pathway that is independent of SNAP-25 cleavage; however, this pathway has not yet been identified.

cAMP has been shown to regulate neurite outgrowth

Cyclic adenosine monophosphate (cAMP) is a second messenger that is generated from ATP by adenylyl cyclase and activates protein kinase A (PKA), which phosphorylates a variety of selected target proteins (Figure 1.6). The cAMP-dependent signaling pathway controls many cellular processes. In neurons, cAMP appears to regulate neurite outgrowth[12]. Several studies have demonstrated that the activation of this pathway blocks the actions of myelin-associated inhibitors, which prevent adult neurons from regenerating after injury[13,14,15]. These results are further supported by another study that concluded that cAMP controls the developmental loss in the ability of neuronal axons to regenerate[16]. Compared with adult neurons, which cannot regenerate axons, neonatal neurons, which can, had higher cAMP levels. Inhibition of PKA in neonatal neurons *in vivo* also reduced the ability of axons to regenerate. Furthermore, the direct

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injection of cAMP into the cell bodies of dorsal root ganglia stimulated regeneration[17].

Collectively, the results of these studies suggest that cAMP is an important regulator of neurite
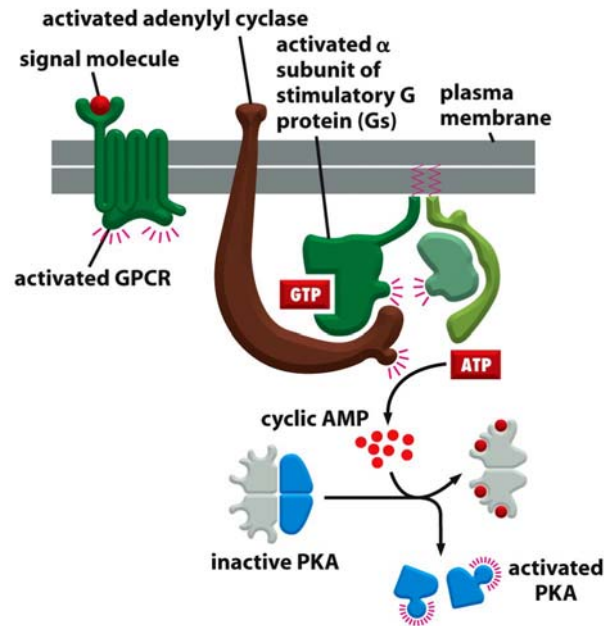
outgrowth.

Figure 1.6: cAMP-dependent intracellular signaling pathway[18]**.** Once an extracellular signal
molecule binds to its transmembrane G-protein-coupled receptor (GPCR), the α subunit of G_s
activates adenylyl cyclase, which then converts ATP to cAMP and pyrophosphate. Increased
cAMP concentration in the cytoplasm activates PKA, which phosphorylates and thereby
regulates the activity of other selected target proteins.

This study sought to identify the intracellular signals BoNT/A activates to stimulate

neurite outgrowth in eMNs. Because of the importance of cAMP in regeneration, it was

hypothesized that BoNT/A promotes neurite outgrowth via cAMP-dependent intracellular

signaling. To test this hypothesis, primary cultures of eMNs were exposed to BoNT/A with and

without several inhibitors of adenylyl cyclase and PKA. In the presence of the inhibitors, the

ability of the toxin to induce sprouting was eliminated, suggesting that the previously

unidentified pathway activated by the toxin is cAMP-dependent. In addition, preliminary

experiments were performed to examine whether the other serotypes of BoNT induce sprouting. The three serotypes tested, BoNT/B, C, and E, exhibited significant neurotrophic activity, but in varying amounts. Additional studies are necessary to complete the characterization of the neurotrophic action of this potent toxin.

CHAPTER 2
MATERIALS AND METHODS

Mice, toxins, and inhibitors

Timed-pregnant NIH Swiss mice were purchased from Harlan Laboratories (Indianapolis, Indiana). All procedures involving animals were approved by the Institutional Animal Care and Use Committee. BoNT/A, B, C, and E were purchased from Metabiologics (Madison, Wisconsin) and used at 10^{-9} M. SQ 22,536, an inhibitor of adenylyl cyclase, and KT 5720, an inhibitor of PKA, were purchased from Sigma-Aldrich (St. Louis, Missouri).

Cell culture

This procedure was modified from an earlier protocol[19]. After embryos were collected from NIH Swiss mice within the thirteenth and fourteenth days of gestation, the embryonic spinal cords were isolated and minced. This tissue was dissociated in 0.025% trypsin for 23 min at 37°C and then removed of the debris by centrifugation through 4% bovine serum albumin (BSA) for 10 min at 300 g. To enrich the cell culture with motor neurons, this suspension was loaded onto a gradient of 6.7% Histodenz (Sigma-Aldrich, St. Louis, Missouri), which was then spun for 10 min at 500 g. The band containing neurons was collected and removed of the remaining debris by further centrifugation through 4% BSA for 10 min at 300 g. The neurons were resuspended in an L-15 medium supplemented with 10% glucose, 2 mM L-glutamine, 1% penicillin-streptomycin, 2 μM progesterone, 5 μg/ml insulin, 100 μg/ml putrescine, 100 μg/ml conalbumin, 3 μM sodium selenite, 7.5% sodium bicarbonate, and 5% horse serum.

Outgrowth assay

The freshly cultured eMNs were deposited on multiwell plates coated with poly-D-lysine and laminin (BD Biosciences, Bedford, Massachusetts) at a density of 10^5 cells/ml and then incubated at 5% CO_2 and 37°C. The cells were treated with cAMP-pathway inhibitors and BoNT 2 and 4 h after plating, respectively, and returned to the incubator for 24 or 48 h after the addition of the toxin. When treatment was complete, the cells were fixed with 4% paraformaldehyde at pH 7.4 for 30 min, washed thoroughly with phosphate buffered saline, and blocked with 5% goat serum for 30 min. After blocking, the eMNs were labeled with a rabbit polyclonal antibody to protein gene product 9.5 (1:300; AbD Serotec, Raleigh, North Carolina), which was then detected with a fluorescent antirabbit secondary antibody.

Measurements and statistical analyses

Using pictures taken of the eMNs by a CCD camera connected to an Olympus inverted microscope, neurite length and the number of primary and secondary branches were measured for each different treatment. Neurite length was defined as the sum of the lengths of all processes on one neuron. Primary branches were defined as processes that sprout directly from the cell body of a neuron; secondary branches were defined as processes that sprout directly from the primary branches of a neuron. Two-tailed *t*-tests were used to determine statistical differences between controls (unmedicated cells) and the other treatments.

CHAPTER 3
RESULTS

**BoNT/A, B, C, and E promote neurite outgrowth**

In the initial experiments, the effects of four different serotypes of BoNT were examined on the neurite outgrowth of eMNs. After plating, the cells were allowed to attach to the plates for 4 h before the addition of the toxin, and at this time, it was visually confirmed that a large number of cells had successfully attached. Each serotype stimulated outgrowth. Compared with time-matched, untreated control neurons, neurons treated with either BoNT/A or B for 24 or 48 h exhibited increased neurite length and an increased number of branches (Figure 3.1). Likewise, similar results were obtained for neurons treated with either BoNT/C or E for 24 h (Figure 3.2). Data is not presented for 48 h for the latter experiment because the cells did not survive. To compare the effects of all four serotypes, the measurements for the toxin-treated neurons were calculated as percent increases from the controls (Table 3.1). At 24 h, BoNT/C induced the largest effect on neurite outgrowth, followed by BoNT/A.

Table 3.1: Neurite outgrowth of motor neurons treated with BoNT/A, B, C and E. Neurite length, the number of primary branches, and the number of secondary branches were averaged for thirty nerve cells exposed to one serotype for 24 h. Data are expressed as the percent increase from the control.
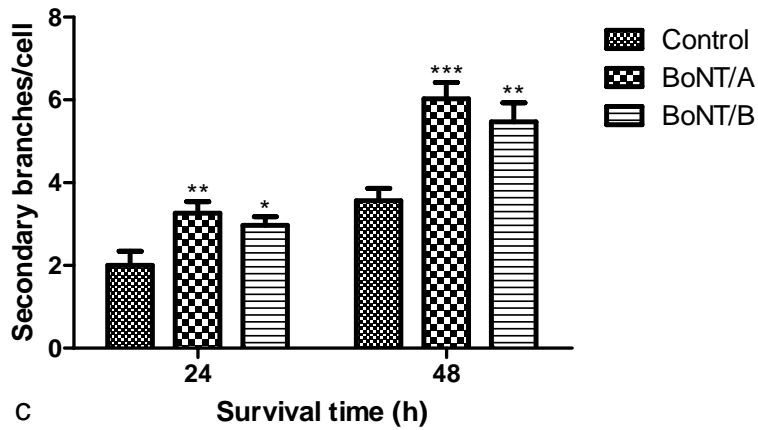
	Neurite length (%)	Primary branching (%)	Secondary branching (%)
BoNT/A	59	21	63
BoNT/B	48	19	48
BoNT/C	70	43	60
BoNT/E	48	40	56
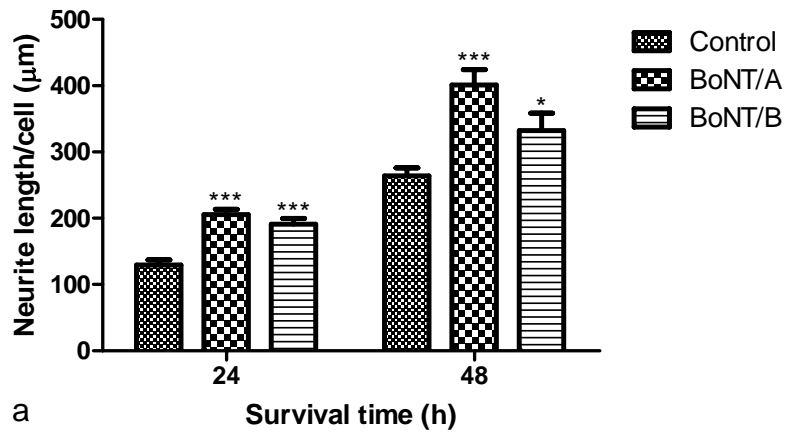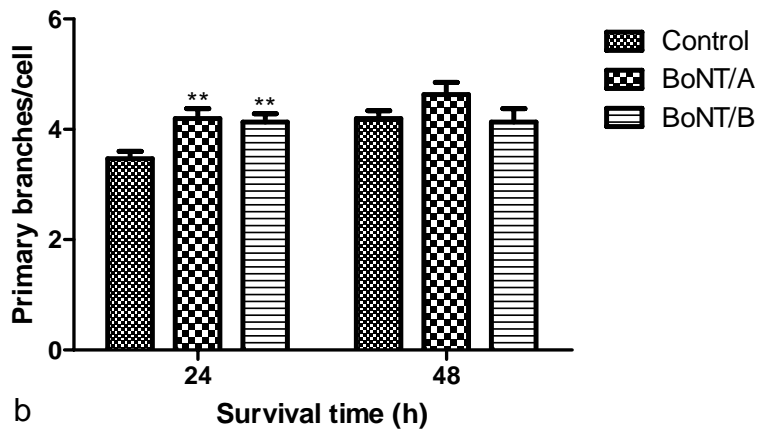
Figure 3.1: Neurite outgrowth of motor neurons treated with BoNT/A and B. Neurite length (a), the number of primary branches (b), and the number of secondary branches (c) were measured. Data are expressed as the mean plus SEM for thirty nerve cells. Asterisks denote significant statistical differences from the control (*** $p<0.001$, ** $p<0.01$, * $p<0.05$). One independent repeat yielded similar results.
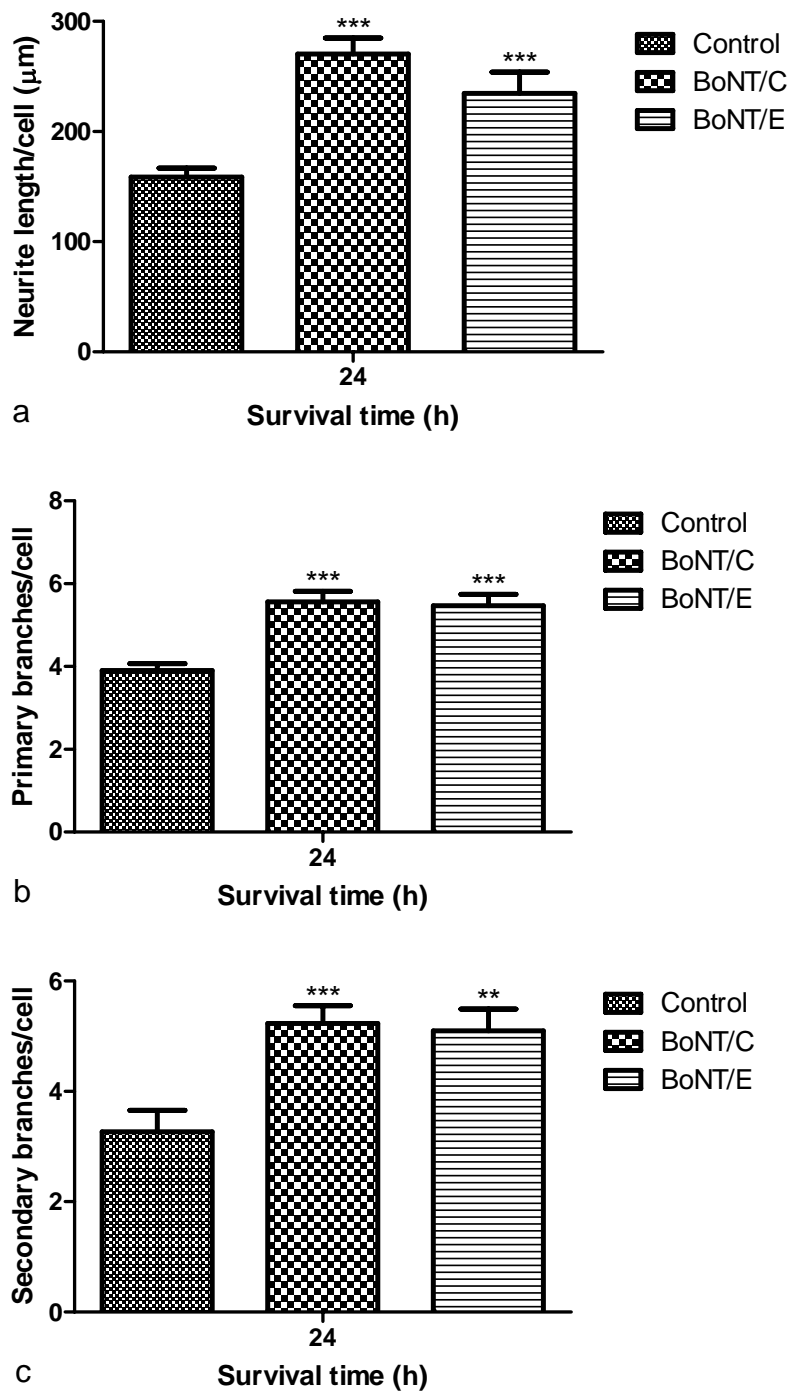
Figure 3.2: Neurite outgrowth of motor neurons treated with BoNT/C and E. Neurite length (a), the number of primary branches (b), and the number of secondary branches (c) were measured. Data are expressed as the mean plus SEM for thirty nerve cells. Asterisks denote significant statistical differences from the control (*** $p<0.001$, ** $p<0.01$).

Inhibition of adenylyl cyclase and PKA eliminates BoNT/A-induced neurite outgrowth

To examine the role of cAMP in sprouting, two inhibitors of the cAMP-dependent pathway were used: SQ 22,536, which inhibits adenylyl cyclase, and KT 5720, which inhibits PKA. The inhibitors were added 2 h before the addition of the toxin and only after visual confirmation of cell attachment. One group of eMNs in each experiment was exposed only to BoNT/A to ensure that a toxin-induced effect was still present, and another group was exposed only to the inhibitor to ensure that inhibition did not reduce basal neurite outgrowth. Both inhibitors eliminated the ability of BoNT/A to induce sprouting. In the presence of SQ 22,536, the neurite length of cells treated with BoNT/A and SQ 22,536 was reduced to the neurite length of control neurons (Figure 3.3). Similar results were obtained for the number of secondary branches; however, primary branching was relatively unaffected. In the presence of KT 5720, there was no statistical difference for neurite length or secondary branching between the controls and the neurons treated with both the inhibitor and the toxin (Figure 3.4). Primary branching was again relatively unaffected. In these two experiments, the inhibitors were added at two different concentrations to determine whether their effects were dose-dependent, but noticeable differences were not obtained between the two concentrations used for each inhibitor.
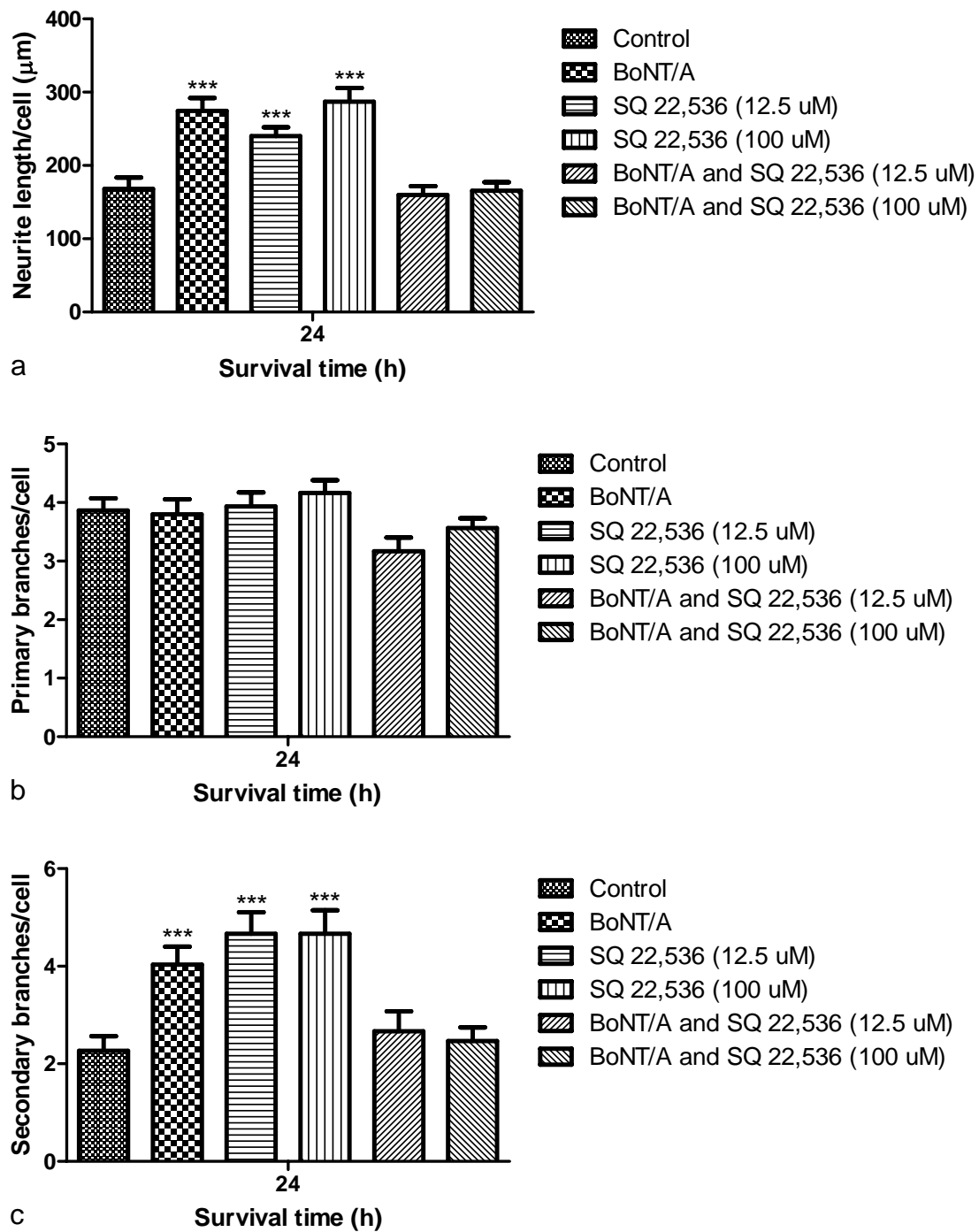
Figure 3.3: Neurite outgrowth of motor neurons treated with BoNT/A and SQ 22,536, an inhibitor of adenylyl cyclase. Neurite length (a), the number of primary branches (b), and the number of secondary branches (c) were measured. Data are expressed as the mean plus SEM for thirty nerve cells. Asterisks denote significant statistical differences from the control (*** $p < 0.001$).
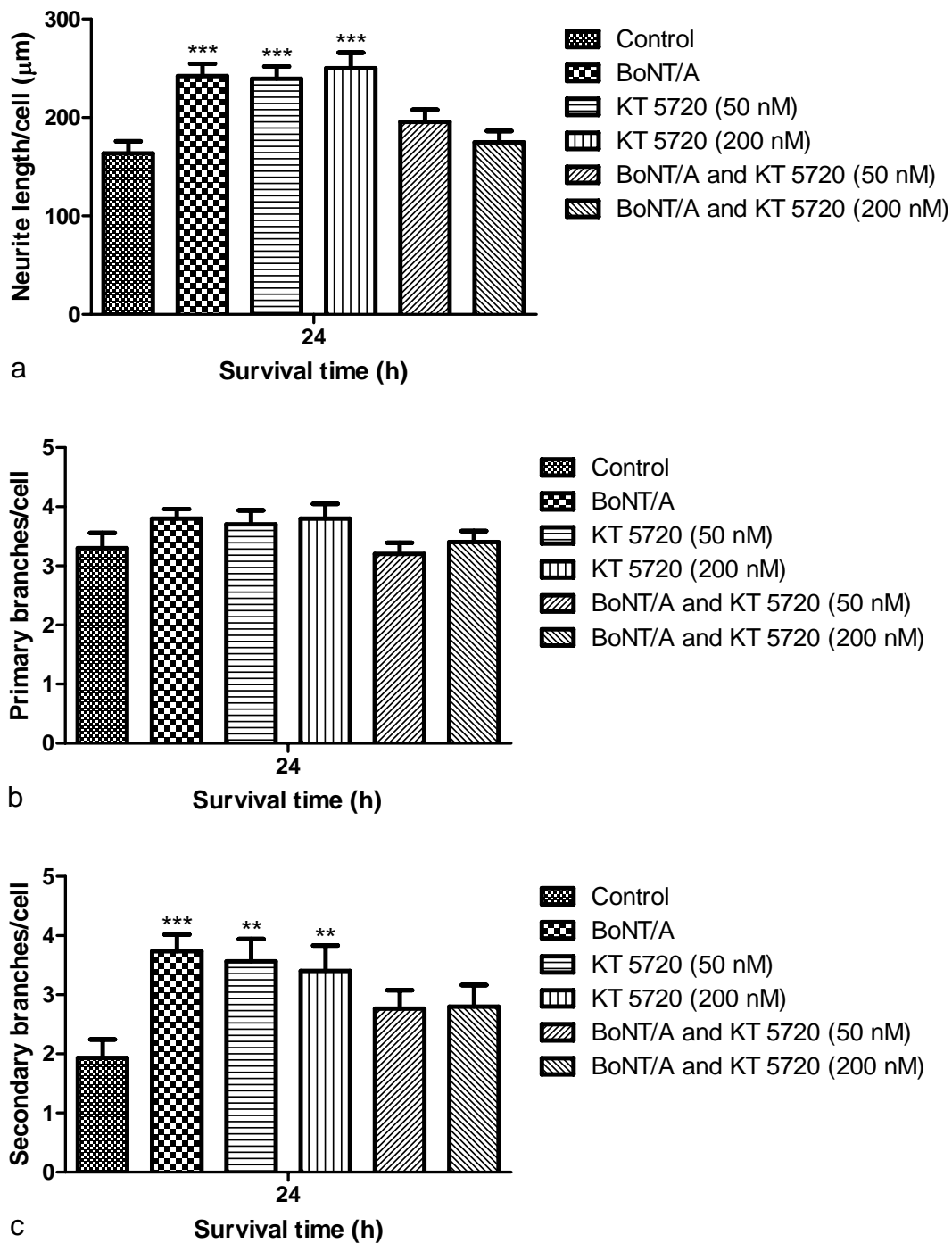
Figure 3.4: Neurite outgrowth of motor neurons treated with BoNT/A and KT 5720, an inhibitor of PKA. Neurite length (a), the number of primary branches (b), and the number of secondary branches (c) were measured. Data are expressed as the mean plus SEM for thirty nerve cells. Asterisks denote significant statistical differences from the control (*** $p<0.001$, ** $p<0.01$).

CHAPTER 4
DISCUSSION

BoNT, the most poisonous substance known, has received much attention in recent decades not only for its potential use as a biological weapon but also for its ability to treat neuromuscular disorders. Although its paralytic action has been well characterized, its ability to stimulate neurite outgrowth is not fully understood. A number of studies have focused on this phenomenon, but until recently no attempts have been made to determine the molecular mechanisms that are responsible for stimulated outgrowth. One study recently suggested that this neurotrophic action of BoNT/A is an indication of previously unrecognized intracellular signaling activity[11]. Although this activity appears to be receptor-mediated, the specific signaling pathway activated by BoNT/A to induce sprouting remains undetermined.

The purpose of this current study was to identify the signaling pathway the toxin activates to stimulate neurite outgrowth. Because cAMP has been shown to control regeneration of axons after injury, it was hypothesized that the binding of BoNT/A to the neuronal membrane triggers a cAMP-dependent intracellular signaling pathway, resulting in additional sprouting. By inhibiting adenylyl cyclase and PKA in two separate experiments, BoNT/A-induced sprouting could be eliminated. These results suggest that BoNT/A promotes neurite outgrowth of eMNs via cAMP-dependent intracellular signaling, thus supporting the hypothesis. To confirm this conclusion, additional studies might attempt to inhibit BoNT/A-induced sprouting by increasing cAMP phosphodiesterase, the enzyme that degrades cAMP. In addition, preliminary

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experiments in this study revealed that BoNT/B, C, and E also induce sprouting; however, whether these other serotypes utilize cAMP-dependent signaling has not been addressed.

Although it can be concluded that BoNT/A activates a cAMP-dependent signaling pathway, these results bring up additional questions about the neurotrophic action of this potent toxin. Of immediate interest is the identification of the downstream effectors of PKA. One possibility is Erk, a protein kinase at the end of a three-component signaling pathway (Figure 4.1). Initial studies have suggested that Erk is an important kinase for outgrowth. For example, it has been shown, along with calcium and cAMP, to regulate the ability of axons to regenerate growth cones[15]. Whether Erk plays a similar role in stimulated outgrowth remains undetermined. Examining the phosphorylation of Erk, as well as Raf and Mek, in BoNT/A-induced sprouting is the next step in identifying potential downstream effectors.
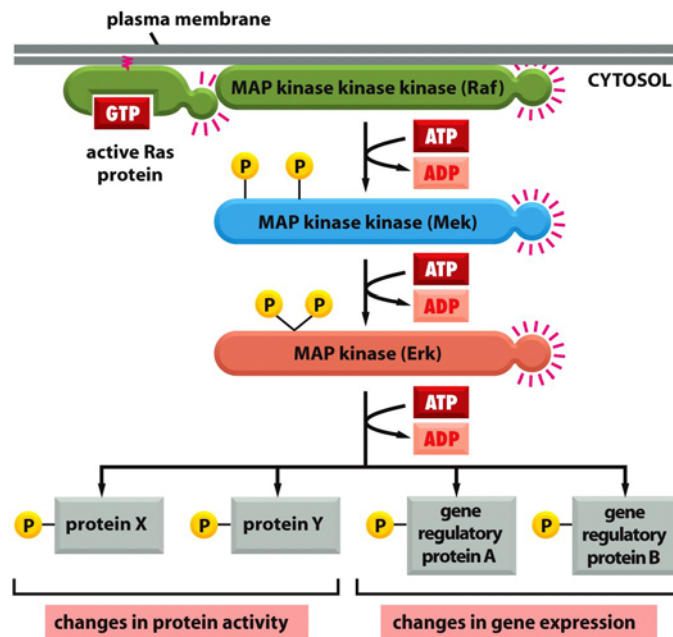

Figure 15-60 Molecular Biology of the Cell 5/e (© Garland Science 2008)

Figure 4.1: Erk intracellular signaling pathway[18]**.** Ras activates Raf, a MAP kinase kinase kinase, at the plasma membrane. Raf then activates Mek, a MAP kinase kinase, which in turn activates Erk, a MAP kinase. Erk phosphorylates and thereby regulates the activity of other selected target proteins, usually in the nucleus.

Because neurodevelopment is highly controlled and the factors mediating growth are rapidly changing in perinatal mammals, it is also important to study BoNT/A-induced sprouting at later stages of development. Primary cultures of motor neurons from postnatal mice, for instance, could be exposed to the toxin to determine whether stimulated outgrowth occurs and, if so, how. Since neuronal cAMP levels drop significantly in neonates[16], it would be interesting to see whether the toxin is capable of raising cAMP to a level where axons can regenerate.

Another area for investigation is the role of the protein receptors for the seven serotypes of BoNT in stimulating outgrowth. Only the protein receptors for BoNT/A, B, E, and G have been identified, and little research has been done to examine the intracellular-signaling capabilities of these receptors. Interestingly, BoNT/A and E share the same receptor, SV2[20]. Whether any similarities exist in their abilities to stimulate outgrowth is yet to be addressed.

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