MOLECULAR MECHANISMS OF BOTULINUM NEUROTOXIN-INDUCED NEURITE OUTGROWTH IN MOTOR NEURONS DERIVED FROM STEM CELLS

by

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(Under the Direction of Julie Coffield)

ABSTRACT

Botulinum neurotoxin serotype A (BoNT/A), known to produce neuromuscular paralysis (botulism) with extremely high potency is also a well-established therapeutic agent used to alleviate the pain and dysfunction associated with a number of neurologic and muscular disorders. Independent of the cellular mechanism that causes botulism, BoNT/A has been found to promote neurite outgrowth of motor neurons. The cellular mechanism(s) underlying this phenomenon remain unknown. In this dissertation, we use HBG3 embryonic stem cell-derived motor neurons (MNs) as an in vitro model to explore mechanisms of BoNT/A-induced neurite outgrowth. The results showed first that the pharmacological inhibitor U0126, which blocks activation of the extracellular signal-regulated kinases 1/2 (ERK1/2), reduced total neurite length and secondary branch formation, while primary neurite formation was not significantly altered. Brain-derived neurotrophic factor (BDNF) enhanced ERK1/2 phosphorylation and promoted secondary neurite formation and increased total neurite length, without changing primary neurite numbers. These findings indicate that BDNF induced ERK1/2 activation is one pathway mediating neurite development in HBG3-derived MNs. BoNT/A stimulated primary and secondary branch formation as well as total neurite length in HBG3-MNs, similar to previously published findings

for mouse primary MNs. The phosphorylation of ERK1/2 was enhanced transiently in response to BoNT/A. Blocking ERK1/2 activation reduced the stimulatory effects of BoNT/A, whereas blocking the activity of AKT or classical protein kinase C isoforms did not. Moreover, RT-PCR microarray analyses revealed that *bdnf* expression was elevated in response to BoNT/A exposure in cultured embryonic primary MNs, and quantitative PCR confirmed the upregulation of *bdnf* in HBG3-MNs. Inhibition of the ERK1/2 pathway blocked the BoNT/A-induced *bdnf* upregulation. The BDNF scavenger TrkB-Fc showed nearly complete blockade of the stimulatory effect of BoNT/A on primary and secondary neurite formation, as well as total neurite length. Taken together, these data suggest that ERK1/2 signaling is critical to mediate secondary neurite branching during development, particularly when stimulated by the neurotrophin BDNF. BoNT/A induces secondary neurite formation through enhancing ERK1/2 activity to up-regulate *bdnf* expression, while the mechanism(s) for basal and toxin-stimulated primary branch formation is not fully determined.

INDEX WORDS: BoNT/A, MNs, neurite outgrowth, ERK1/2, BDNF

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## Introduction

Botulinum neurotoxins (BoNTs) are biological poisons well known for their extremely high potency as well as for both their noxious and beneficious properties. BoNTs cause botulism, a life-threatening paralytic disease of humans and animals, by inhibiting the release of neurotransmitter at synapses, primarily acetylcholine (ACh) at the neuromuscular junction (Simpson, 2004; Erbguth, 2008). Among the seven distinct serotypes of BoNT (A-G) and the recently proposed serotype H, types A, B, E and F are the major ones affecting humans (Smith, 2009; Dover et al., 2014). Although potentially fatal, there is still no cure for botulism and the only effective treatment remains to be long term, supportive and intensive care (Sobel, 2005). On the other hand, therapeutic applications of BoNTs keep expanding, and include usage not only for cosmetic purposes (BOTOX®), but also for various dystonia and spasticity disorders, autonomic disorders and pain (Jankovic, 2004). Interestingly, a common "side effect" of BoNT exposure known as sprouting impacts both the onset of recovery from botulism and its dosing frequency when used as a therapeutic drug. Although botulism is potentially reversible, it requires an extended period (3-6 months) for the recovery of synaptic function, which is currently thought to be initiated by neurite sprouting at the poisoned nerve terminals (de Paiva et al., 1999). When used for therapeutic purposes, BoNT serotype A (BoNT/A) needs to be injected repeatedly, usually every few months, since the functional sprouting and subsequent recovery of the affected terminals diminishes the toxin's therapeutic effect (Harrison et al., 2011). Because sprouting plays a critical

role in both recovery and therapeutic efficacy, the importance of studying the cellular mechanisms of sprouting following BoNT/A exposure is clear. Such study could lead to the ability to manipulate neurite sprouting to mimic different conditions; for instance, to stimulate sprouting which could potentially facilitate functional recovery from botulism, or to block sprouting in order to maintain clinical efficacy for a longer period. The studies presented in this dissertation begin to fill the knowledge gap regarding the intracellular pathways that regulate the promotion of neurite outgrowth ('sprouting') by BoNT/A and provide potentially useful intracellular targets for modulation of the sprouting response.

**Specific Aims**

The overall goals of the studies presented in this dissertation were to identify the intracellular signaling pathways mediating BoNT/A-induced neurite outgrowth ('sprouting') using an in vitro model of motor neurons (MNs), and identify potential effector molecules involved in this toxin action. Three specific aims were pursued for these studies:

In **Specific Aim 1**, I assessed the usefulness of HBG3-MNs derived from mouse embryonic stem cell (mESC) for in vitro studies, and examined critical signaling pathways regulating neurite development in these neurons. Based on a review of the published literature, the working hypothesis was that the extracellular signal-regulated protein kinase 1/2 (ERK1/2), phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) pathways regulate neurite development in these MNs. The results of this study are presented and discussed in Chapter 2.

In **Specific Aim 2**, I investigated the signaling pathways involved in BoNT/A-induced neurite outgrowth in HBG3-derived MNs. Particularly, this study examined how BoNT/A modulated the activity of three critical signaling pathways and whether inhibition of their activities affected BoNT/A-induced neuritogenesis. The working hypothesis was that BoNT/A activates

these pathways to promote neurite outgrowth. The results of this study are presented and discussed in Chapter 3.

In **Specific Aim 3**, I investigated the role of the neurotrophin BDNF in BoNT/A-induced neurite outgrowth. The working hypothesis was that BoNT/A up-regulates BDNF expression and secretion, which enhances neurite sprouting and growth in HBG3-MNs via the signaling pathways studied in aim 2. The results of this study are presented and discussed in Chapter 4.

## Background and Significance

### Pharmacokinetics of BoNTs

BoNT is an exotoxin mainly produced by *Clostridium botulinum*, a rod-shaped, gram-positive, spore-forming bacterium that is often found in water sediments and soil (Erbguth, 2008). It causes the disease botulism, which presents as a symmetrical, flaccid paralysis that starts from the face and descends towards the limbs, resulting in respiratory paralysis and may be lethal if not treated (Sobel, 2005). Routes of BoNT exposure include ingestion, inhalation, wound and parenteral exposure (Nantel, 1999). Adults may develop botulism following the ingestion of food contaminated with preformed toxin ('foodborne botulism'); infants may develop botulism after ingesting clostridial spores sometimes found in honey products ('infantile botulism'). The time from exposure to symptom onset can vary from as early as 6 hours to 8 days or even longer, but ranges mostly between 18 to 36 hours (Shapiro et al., 1998). Inhalation botulism has been reported rarely in some laboratory workers, while the generation of toxin-containing aerosol as a bioweapon for terrorist attacks remains a significant concern for Homeland Security (Nantel, 1999). Wound botulism may occurs following injection of illicit drugs (heroin), although there have been cases of wound infection from open bone fractures.

Absorption of BoNT can be categorized based on protective barrier properties. Wound infection occurs via a compromised skin barrier; entry via ingestion and inhalation involves transcytosis of the neurotoxin across intact gut or respiratory epithelium (Simpson, 2013). It is worth noting that epithelium might express different binding sites as neurons do to facilitate BoNT entry, with the evidence that mutation of BoNT/A binding domain kept it from entering neurons but not gut epithelium (Elias et al., 2011).

Once BoNT reaches the blood stream, it is distributed throughout the systemic circulation within minutes (Al-Saleem et al., 2008). The toxin, with a molecule weight of 150 kDa, has a long duration of action, largely because it is highly stable in blood, and can be detected in some patient even 25 days after the acute onset of symptoms (Sheth et al., 2008). BoNT does not cross the intact blood-brain barrier, instead it preferentially targets neuromuscular junctions, binding to MN nerve terminals, with high affinity (Verderio et al., 2006). The holotoxin of BoNT is composed of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) associated by a single disulfide bond (Fig 1.1) (Brunger and Rummel, 2009; Rossetto et al., 2014). The HC binds to surface receptors on neurons and helps in endocytosis of the toxin. Current understanding of BoNT entry follows a dual receptor model: an N-terminal domain on the HC is responsible for binding with proteoglycans, and a C-terminal domain binds to gangliosides. Specifically, BoNT first anchors to high density, low affinity gangliosides on the membrane. Then through lateral movement, BoNT binds to low density, high affinity proteoglycans. Moreover, these binding substrates can be serotype specific. For example, BoNT/A binds to synaptic vesicle protein 2 (SV2) and trisialoganglioside 1b (GT1b), while BoNT/B binds to synaptotagmin and GM3 preferentially. Recently a third receptor for BoNT/A, fibroblast growth factor receptor 3 (FGFR3) has been proposed (Jacky et al., 2013). Both BoNT/A holotoxin and HC serve as agonists to this high

affinity receptor and induce receptor phosphorylation. The expression level of FGFR3 could attribute to the sensitivity of different types of neurons to BoNT/A.

Currently there is no evidence to demonstrate that circulating BoNT is metabolized or eliminated by the liver, kidney or circulating phagocytic cells (Simpson, 2013). The intact holotoxin is essential for the binding, internalization, and translocation of the toxin in the nerve terminal. Once inside target cells, this dichain molecule quickly dissociates into a HC and a LC, which allows the LC to be released and act as a $Zn^{2+}$ metalloprotease (Simpson, 2004; Brunger and Rummel, 2009). A few in vitro studies have reported that the ubiquitin-proteosome system degraded the LCs of different serotypes with various potency (Shi et al., 2009; Tsai et al., 2010). The intracellular degradation of the HC, which is mainly involved in toxin binding, has not been well investigated (Brunger et al., 2007).

**Mechanisms of transmitter release, muscle contraction and botulism**

The process of skeletal muscle contraction under physiological conditions first requires spinal MNs that innervate the neuromuscular junction to be activated (Sudhof and Rizo, 2011). Then the resultant action potential propagates along the axon and opens voltage-gated calcium channels that are localized in the axon terminal and regulate neurotransmitter release. Synaptic vesicles concentrated within the nerve terminal contain neurotransmitter and a subset of these vesicles are 'docked' along the specialized region of the nerve terminal known as the active zone. At the neuromuscular junction, these vesicles contain ACh and the region containing the active zone is known as the endplate. Because of the ion concentration gradient across the axon membrane and the concentrated localization of calcium channels, calcium flows into the nerve terminal in discrete regions and creates $Ca^{2+}$ microdomains near synaptic vesicles in the active zone (Young and Neher, 2009). The distinct rise in $Ca^{2+}$ around the docked vesicles initiates the

fusion of the vesicles with the membrane of the active zone and the release of transmitter into the synaptic space.

The core fusion complex inside nerve terminals is composed of four molecules of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, including one molecule of synaptobrevin, one of syntaxin and two of SNAP25 (Young and Neher, 2009; Hong and Lev, 2014). Synaptobrevin is localized on the vesicle membrane (v-snare), while syntaxin and SNAP25 are on the target membrane (t-snares) or presynaptic membrane. Under resting state, the fusion complex is either unbound, or kept in a hemifused state by the protein complexin (Trimbuch and Rosenmund, 2016). Synaptotagmin, another vesicular protein, acts as a sensor and detects changes in $Ca^{2+}$ concentration in the active zone. When $Ca^{2+}$ enters, it binds to synaptotagmin and complexin is released, allowing v- and t-SNAREs to interact to form a parallel coiled-coil structure. Zippering of this complex provides the driving force for the two sources of lipid bilayer to move closer and fuse with each other (Hong and Lev, 2014). As the two membranes fuse, the ACh inside the vesicles is released, and diffuses across the synaptic cleft to activate receptors on the muscle cell membrane. BoNTs and the functionally-related tetanus neurotoxin (TeNT) enzymatically cleave SNARE proteins to impair synaptic vesicle fusion and neurotransmitter release, with serotype and substrate specificity (Schiavo et al., 2000). For example, BoNT/A and BoNT/B target SNAP25 and synaptobrevin, respectively (Fig 1.1). BoNT/E also cleaves SNAP25, but cleaves at a different site than type A. Without vesicle fusion, $Ca^{2+}$ can no longer trigger ACh release. As a result, there is no ACh to cause contraction, and muscle activity is paralyzed.

Acetylcholine binds to nicotinic ACh receptors on muscle and opens ligand-gated $Na^+$ channels, allowing $Na^+$ to flow into the muscle cell and depolarize the membrane. When this depolarization reaches threshold of activation, an action potential is triggered. This causes the

dihydropyridine receptors on the muscle cell T-tubule to activate, leading to the activation of ryanodine receptors on the intracellular sarcoplasmic reticulum and subsequent $Ca^{2+}$ release. Increased cytoplasmic $Ca^{2+}$ binds to troponin, which initiates actin-myosin binding. Myosin heads execute a power stroke, to help actin filaments slide towards the center of sarcomeres. At this point, the sarcomere shortens and muscle contracts.

**Clinical presentations of botulism and applications of BoNT/A**

Because of the protection provided by the blood-brain barrier, BoNT mainly affects spinal MNs in vivo (Dressler, 2012). The symmetrical, flaccid paralysis that is characteristic of botulism begins in the face and descends towards the limbs (Sobel, 2005). Involvement of cranial nerves causes blurry vision, ptosis, dysphagia and dysarthria. Patients may have anhydrosis, dry mouth, or postural hypotension if the autonomic nerves are blocked. Paralysis of the skeletal muscle may spread to the diaphragm and other accessory muscles for breathing, causing severe respiratory distress. Although potentially recoverable, there is no cure once BoNT is internalized into nerve terminals, and treatment consists of supportive long-term intensive care. On the other hand, most therapeutic utilizations of BoNT/A rely on the blockade of the neuromuscular junction and subsequent target muscle paralysis (Dressler, 2012). For instance, various muscle hyperactivity disorders, such as focal dystonia and spasticity, can be relieved by BoNT/A. Spasticity, sometimes triggered by certain neurological disorders (e.g. cerebral palsy, multiple sclerosis and spinal cord injury), are caused by the loss of inhibition from upper MNs to lower MNs (Sevim et al., 2015; Tilton, 2015). Not surprisingly, BoNT/A has been utilized to treat such neurological conditions successfully. Moreover, pain induced by spasticity is largely reduced by BoNT/A (Gupta, 2006).

Interestingly, several studies reported that neurotransmitter release from other neuronal types, such as sensory and autonomic ganglia neurons, and even neurons within the central nervous

system (CNS) could be affected by BoNT/A with different potencies (Bozzi et al., 2006; Kiris et al., 2011). Specifically, a BoNT/A dose response study using mouse primary cell culture reported that in MNs the lowest toxin dose to cause detectable SNAP25 cleavage was 25 pM, whereas in dorsal root ganglia neurons the lowest dose was 100 pM (Kiris et al., 2011). BoNT/A has been indicated in some cases of pain management (i.e. trigeminal pain, neuropathic pain), as an interaction between toxin and the transient receptor potential cation channel subfamily V member 1 (TrpV1) has been associated with altered nociception and modulation of the expression of inflammatory factors (Matak et al., 2014; Edvinsson et al., 2015; Li and Coffield, 2016). In regards to the treatment of hypersalivation, BoNT/A injection has less adverse systemic effects than anticholinergics and is of lower risk than radiotherapy or resection of the salivary glands (Evidente and Adler, 2010; Dressler, 2012). Moreover, ongoing research in dogs showed that BoNT injection in the epicardial autonomic ganglia suppressed atrial fibrillation that is mediated by the vagal nerve (Oh et al., 2011). Other research has shown that direct injection of BoNT/E into rat hippocampus reduced seizures induced by glutamate agonist (Costantin et al., 2005).

In addition, it has been documented that single BoNT/A injections caused temporary muscle atrophy and this effect has been manipulated to change mandibular or calf muscle contour for cosmetic purposes (Durand et al., 2016). BoNT/A also produced beneficial effects in wound healing and prevention, although the exact mechanisms are not fully understood. For example, one study demonstrated that BoNT/A was protective against decubitus ulcer formation in mice (a cutaneous ischemia-reperfusion model), and it was proposed that this protection might be due to vasodilation caused by inhibition of the sympathetic tone (Uchiyama et al., 2015). BoNT/A is also reported to reduce tension forces on wound edges, prevent keloids/hypertrophic scars, and produce a better appearance after cleft lip repair (Al-Qattan et al., 2013).

Unfortunately, the clinical application of BoNT/A is accompanied by certain adverse effects (Dressler, 2012). Some of the toxin may diffuse from the local injection site to adjacent and distant muscles, which could result in undesired side effects (Whelchel et al., 2004). The duration of action of BoNT/A when applied clinically is temporary, and the desired treatment effects wears off after several months, often necessitating subsequent injections (Rogozhin et al., 2008). This temporary duration of action of BoNT/A can be largely explained by the sprouting response initiated following toxin application.

**Neuritogenesis as a novel toxin effect**

Although not well investigated, BoNT/A-induced neuritogenesis was noticed early in humans as increased terminal sprouting at paralyzed neuromuscular junctions (Holds et al., 1990). Later, in vivo research revealed that these sprouts were actually functional, with active vesicle recycling (de Paiva et al., 1999). This response is thought to be the initiation of recovery from BoNT intoxication. Superfluous sprouts were then eliminated after the parent terminal recovered. Further in vitro studies showed that BoNT/A exposed neurons had not only increased number of primary and secondary neurites, but also increased total neurite length (Bonner et al., 1994; Coffield and Yan, 2009). This highlights the importance of studying the cellular mechanisms for this induced neuritogenesis. It could be ideal to manipulate neurite sprouting to confront different conditions: to stimulate sprouting and facilitate functional recovery from botulism, or to block it in order to delay recovery and maintain its clinical efficacy for a longer period. A few studies indicate that muscle fiber-derived extracellular molecules, like insulin growth factor (IGF), might partly mediate the sprouting process observed in BoNT-paralyzed muscles (Caroni and Schneider, 1994; Harrison et al., 2011). These findings are based on the assumption that sprouting relies on the activity of neighboring muscles. However, one study showed that BoNT/A induces

neuritogenesis in the absence of muscle fibers, implying the significant involvement of some neural factors in this process (Coffield and Yan, 2009). In addition, although intra- and extracellular stimulants trigger signaling transduction, signaling cascades ultimately converge inside neurons. To date, no reports exist on BoNT/A related neuronal signaling profile.

**Mechanisms of neuritogenesis**

Neuritogenesis requires a sophisticated interplay of actin and microtubules as well as many adaptor proteins (Mattila and Lappalainen, 2008; Stiess and Bradke, 2011; Sainath and Gallo, 2015). Although the exact mechanisms for neurite outgrowth are not clear, filopodia and neurite formation share many similarities and the model for filopodia formation is well accepted. The first step of formation is the generation of highly-dynamic, thin protrusions composed of actin bundles. The G-actin is constantly incorporated into the barbed end whereas subunits disassemble at the opposite end, forming a treadmilling of actin filaments and pushing the protruding membrane forward. Actin filament branching uses several adaptor proteins, one of which is actin-related protein 2/3 complex (ARP2/3). ARP2/3 acts like a seed binding to the mother filament and allows new filament to extend away from it. Extension and stabilization of microtubules into the growth cone elongates the neurites and delivers organelles into the newly formed neurites.

Neurite outgrowth is an overall response from attractive/repulsive and long-range/short-range cues in the environment (Thiede-Stan and Schwab, 2015). Among the extracellular factors that have been reported, neurotrophins promote whereas myelin-associated growth inhibitors block neurite outgrowth. Effects of netrins, some ephrins and semaphorins can be either permissive or suppressive, depending on the concentration and receptor composition on neurons. Among all stimulatory molecules, neurotrophins are the most well-known supportive factors for neurons (Huang and Reichardt, 2001). They are involved in neuronal survival and development, including

neuritogenesis. Based on their gene sequence and structure, current members in the neurotrophin family are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Other factors, such as glial cell-derived neurotrophic factor (GDNF), although not present in the neurotrophin family, have demonstrated roles in the development and maintenance of the nervous system. Sources of neurotrophins mainly are the post-synaptic targets of innervation and occasionally neurons through autocrine and paracrine regulations. In case of injury, inflammatory cells, such as macrophages and mast cells, can contribute to neurotrophin secretion by releasing certain cytokines. Neurotrophins have two receptors. One is the low-affinity receptor, p75NTR, which is in the tumor necrosis factor receptor family and shares with it a death domain in its structure. Activation of this receptor determines the survival of the neuron during development. The other receptor family is the tropomyosin-related kinases (Trk) that has three members. NGF binds to TrkA, BDNF and NT-4 bind to TrkB, and NT-3 binds to TrkC. Ligand binding stimulates dimerization of these receptors, followed by subsequent receptor phosphorylation at the cytoplasmic domain by tyrosine kinases.

Interestingly, the function of neurotrophins varies across neuron types. BDNF has been suggested to be critical for the neuritogenesis of spinal sensory neurons, such as dorsal root ganglion cells (Liu et al., 2016). Their actions are mediated through the ERK1/2 pathway, rather than the phosphatidylinositol 3- PI3K or phospholipase C (PLC) pathways. Activation of the ERK1/2 pathway further activates transcription factors Etv4 and Etv5, followed by enhanced expression of growth-associated protein-43 and neurofilament protein. Similarly, BDNF also exhibits neuritogenic effects on spinal MNs (both during development and after injury) and corticospinal neurons (Hollis et al., 2009; Wilhelm et al., 2012; Wang et al., 2015). However, pharmacological application of exogenous BDNF is limited because of its short half-life and low

permeability across blood-brain barrier (Poduslo and Curran, 1996). Therefore, significant research effort has been focusing on enhancing endogenous BDNF expression, developing new delivery system or fusion proteins (Johnson and Koria, 2016; Wang et al., 2016). NGF, on the other hand, regulates sensory and sympathetic ganglions, but does not have any trophic effect on MNs (Yan et al., 1988; Yan et al., 1992).

The currently accepted theory to explain the diverse types of regulation by neurotrophins centers around the differences in receptor composition found on neurons (Thiede-Stan and Schwab, 2015). Despite the diversity of receptor composition, their cognate stimulated signal transduction pathways are relatively restricted. Most interestingly, neurotrophins activate similar intracellular signaling pathways as other mitogens (Huang and Reichardt, 2001). Specifically, neurotrophin induced neuritogenesis often involves PI3K, PLC and ERK1/2 pathways (Duman and Voleti, 2012). First the neurotrophins bind to cell membrane Trk receptors and trigger their dimerization and autophosphorylation. Through adjacent adaptors, the p-Trk can activate PI3K and further phosphorylates its downstream kinase AKT; the p-Trk can also activate PLC, which leads to the activation of protein kinase C (PKC); p-Trk is able to trigger the small GTPase Ras to switch to an "on" state by binding to GTP, and activate downstream Raf-ERK1/2 kinases. ERK1/2 belongs to the mitogen-activated protein kinases (MAPK) family (Cargnello and Roux, 2011) (Fig 1.2). Other MAPK family members, c-Jun N-terminal kinases (JNKs) and p38, are also reported to be involved in neuritogenesis, which is independent of ERK1/2 kinases function (Barnat et al., 2010; Mullen et al., 2012). ERK5 is also reported to have neuritogenic functions, although it's hard to separate them from ERK1/2 because of the large sequence homology (Obara et al., 2009).

The cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA) pathway could also promote neurite outgrowth and is usually activated by G-protein coupled receptors (GPCRs)

(Aglah et al., 2008; Shelly et al., 2010; Stiles et al., 2014). Pituitary adenylate cyclase-activating polypeptide, which is a GPCR ligand and widely expressed in the nervous system during development, promotes neurite outgrowth by activating the cAMP pathway (Stiles et al., 2014). It has also been reported that increased cAMP can induce neurite outgrowth through some other signaling molecule that is PKA independent, such as exchange protein activated by cAMP (Murray and Shewan, 2008). In addition, cAMP induction can occur through intracellular signaling crosstalk. For example, ERK can regulate the activity of phosphodiesterase, which is responsible for cAMP degradation (Johnson and Lapadat, 2002); $Ca^{2+}$ can also induce cAMP production, either through activating adenylyl cyclase, or through $Ca^{2+}$-dependent proteins. Moreover, the Trk receptor can be transactivated by GPCR ligands, although how exactly these two receptors interact to contribute to neurite outgrowth is not clear yet (Rajagopal et al., 2004).

Although a number of studies indicated that these pathways have significant roles in neuritogenesis, only a few were done in MNs and with somewhat variant results (Perron and Bixby, 1999; Larsson, 2006; Read and Gorman, 2009). The ERK pathway mediated fibroblast growth factor induced neurite extension and axon guidance, whereas in cAMP-induced neuritogenesis ERK was not activated (Aglah et al., 2008; Soundararajan et al., 2010). AKT has been associated with adult MN regeneration, although the exact downstream targets which differentiate the role from other cellular responses are less investigated (Namikawa et al., 2000; Read and Gorman, 2009). PKC has been shown to have complex functions. It seems to mediate neurite elongation in MNs; conversely, inhibitory factors in the CNS, like chondroitin sulfate proteoglycans, are also known to act via the PKC pathway (Guzman-Lenis et al., 2009; Usher et al., 2010).

Rho/Rac1/Cdc42 are members of the small Rho GTPases (Tsuji et al., 2002). They don’t have a membrane bound receptor, and their activation could be possibly through intracellular

crosstalk or though the release of Rho-GDP dissociation inhibitors. Typically, low concentration of Rho activates mDia, which could further activate Rac1; high concentration of Rho activates ROCK, which inhibits Rac1 activation. These opposing effects controls Rac1 activity at specific subcellular compartments, leading to either elongation or retraction through regulating the dynamics of actin filaments.

Besides the above mentioned signaling pathways, there are some other pathways that have been reported to play important roles in neuritogenesis. As an example, neuropoietic cytokines bind to glycoprotein 130 (gp130) receptor, activate Janus kinases (JAK) which are known as non-receptor tyrosine kinases, and stimulate downstream signal transducers and activators of transcription (STAT) (Quarta et al., 2014). There may be crosstalk between JAK-STAT with MAPK pathways, since gp130 activates both. Bone morphogenetic protein (BMP), by activating the downstream Smad1 pathway, also regulates axon regeneration in sensory neurons (Parikh et al., 2011). Whether these pathways are cell type specific or triggered only at particular pathophysiologic conditions needs to be further studied.

The cellular targets of the above cascades can be classified into two categories roughly. One is the cyclic AMP-response element binding (CREB) transcription factor. The activated kinases, like p-PKA and p-ERK1/2 translocate into the nucleus, phosphorylate CREB and recruit CREB binding protein to the promoters of cAMP responsive genes (Zhang et al., 2005). Although the target genes in the nervous system are not fully revealed, BDNF and GDNF are two known targets which are beneficial for MN development (Lonze and Ginty, 2002; Cen et al., 2006). The other target is cytoskeleton as mentioned earlier. Rho GTPases can directly regulate actin filament and microtubule polymerization, which further contribute to neurite outgrowth (Auer et al., 2011). For example, RhoA, by activating its effector ROCK, controls profiling activity and actin stability

thereafter (Da Silva et al., 2003). Clostridium botulinum C3, an ADP-ribosyltransferase known to inactivate RhoA, has been used to stimulate neurite outgrowth (Auer et al., 2012). PKC is reported to mediate the growth cone collapse, possibly through regulating microfilament modulators such as Adducin, Fascin and Vinculin (Larsson, 2006). Intergrin is not directly involved in neuritogenesis, but it can stabilize the extensions by making cell-matrix contact. PKC is thought to regulate integrin recycling, which also affects neurite extensions. Another cytoskeleton component is the microtubule, whose regulators are also reported to be controlled by these signaling cascades. Theoretically, synthesis of membrane components, like lipids, can be another downstream of signaling cascades, although it is not quite correlated with neuritogenesis so far.

The regeneration capability differs between CNS and peripheral nervous systems (PNS). For instance, dorsal root ganglion neurons have two axonal processes, one extends peripherally and one goes into the spinal cord. Research has shown that axons can grow into the dorsal column following sciatic nerve injury but not into the spinal cord if the dorsal column is transected (Neumann and Woolf, 1999). Inhibitory factors of CNS regeneration include the myelin associated inhibitors (e.g. Nogo-A, myelin-associated glycoprotein, ephrin-B3) produced by oligodendrocytes in the CNS, and the chondroitin sulfate proteoglycans (e.g. neurocan, versican, brevican) found in glial scars (Huebner and Strittmatter, 2009). Besides the composition of the extracellular environment, regeneration-associated genes (RAGs) could also contribute to the differences in regeneration between CNS versus PNS. Examples of such genes include transcription factors like c-Jun, and cytoskeletal proteins like GAP-43. Following injury, the RAG expression level in PNS neurons is high whereas that in CNS neurons are very modest.

**Selection of In Vitro Models for BoNT Studies**

Since BoNTs preferentially target spinal MNs, it is highly desirable, whenever possible, to pursue toxin studies using MN preparations. Preparations from human tissues and those that mimic the neuromuscular junction would be ideal. However, access to such preparations is severely limited and technically challenging. While this preparation has been very useful in mechanistic studies of BoNT action, its complexity makes it impractical for investigative studies of intracellular signaling. Therefore, for the studies in this dissertation it was first necessary to identify optimal in vitro approaches to study neuritogenic mechanisms of BoNT/A in MNs. A number of currently utilized in vitro models exist and include primary MNs isolated from rodents, hybrid neuronal cell lines, and stem cell-derived MNs from human or mouse (Gordon et al., 2013). Primary neurons, isolated directly from the ventral spinal cord, are most desirable as they most closely recapitulate MNs in vivo. In fact, preliminary studies performed in support of this dissertation utilized mouse embryonic spinal neurons. However, for the signaling pathway studies in particular, this preparation proved to be quite problematic for a number of reasons, not the least of which was that differentiated primary neurons in culture are post-mitotic cells that do not replicate, therefore limiting the number of cells that can be harvested from each animal. This necessitated the use of a large number of animals. Furthermore, the labor-intensive procedures required to isolate and culture the neurons significantly delayed research progress. Thus, the need for sophisticated isolation and purification techniques coupled with the financial and ethical costs of using large numbers of animals all pointed to a need to find a better model. Although the proliferative nature of transformed hybrid cells expand cell numbers easily, their capacity to exhibit unfavorable features not associated with true neurons made them a less desirable option (Gordon et al., 2013). An alternative consideration was to use stem cells or neuroepithelial cells, which are self-renewable and have the potential to differentiate into MNs. Differentiation of

induced pluripotent cells generally requires longer periods of time and more defined culture conditions, compared to the process using mouse stem cells (Wichterle et al., 2002; Qu et al., 2014).

The differentiation of MNs in general has been characterized (Wichterle et al., 2002). Briefly, neural induction starts from a region of ectoderm in the embryo forming the neuroepithelium and later the rostral brain. Retinoic acid (RA) induces a caudalizing signal and drives these neural progenitors to take a spinal position. Then the spinal progenitors can take a ventral position under the influence of sonic hedgehog (Shh) and develop into MNs. Of course, the actual process in vivo is much more complicated and requires precise control by different morphogens. For example, the process of dorsal-ventral positioning in the spinal cord has been described (Lewis, 2006). There are many different classes of neurons in the spinal cord and their fates are determined by the expression of certain combination of transcription factors. Bone morphogenetic proteins (BMPs) and Shh, produced from surface ectoderm and notochord, respectively, specifies the dorsal-ventral polarity. In the ventral spinal cord, transcription factors are categorized into two groups, class I and class II. Their expression depends on the Shh signaling, either promoting or inhibiting, and cross repression from each group. Combinations of different concentrations of transcription factors specifies the domains for the five neuronal populations. Similarly on the dorsal side, different combinations of transcription factors determine the fate and boundaries of the nine neuronal populations.

HBG3 cells are a line of mESCs that can be successfully differentiated into MNs at a high ratio and possess several characteristics that seem ideally suited for BoNT studies (Wichterle et al., 2002). For example, an *eGfp* gene is incorporated into the genome and they spontaneously express enhanced green fluorescent protein (GFP) under the control of the *HB9* promotor, a transcription factor and post-miotic marker of differentiated MNs. Similar to primary mouse MNs,

the HBG3-derived MNs have been shown to express choline acetyltransferase (an enzyme critical for the synthesis of ACh), the SNARE protein SNAP-25 (the intracellular target of BoNT/A), and the vesicular protein synaptic vesicle glycoprotein 2A (previously identified as the membrane receptor for BoNT/A) (Kiris et al., 2011). HBG3-MNs have also been reported to populate into the spinal cord and innervate target muscles following reintroduction (Wichterle et al., 2002). These features, as well as their demonstrated sensitivity to BoNT intoxication previously reported by Kiris et al, indicated that HBG3- MNs may be useful candidates to study the neuritogenic mechanisms of BoNT/A.

**Preliminary Studies**

Initial studies done in support of this dissertation project started with examining the signaling alterations in primary cultures of mouse embryonic MNs after BoNT/A exposure. Previous studies in the Coffield lab had used mouse embryonic primary MNs to examine the effect of BoNT/A on neurite outgrowth (Coffield and Yan, 2009). Thus it was desirable to use the same preparation to analyze potential signaling pathways mediating this toxin action. In these initial experiments, the phosphorylation of ERK1/2, AKT and GSK3β in the presence of BoNT/A plus GT1b was examined. GT1b, the low affinity ganglioside receptor was added to enhance BoNT/A uptake. Sample immunoblots depicting protein phosphorylation are included in figure 1.3 and 1.4. Because of the presence of non-specific banding and the lack of time matched, as well as missing loading controls in these initial experiments, it was necessary to repeat the experiments several times to optimize the experimental protocol. These initial experiments were inconclusive and at this point it became clear that while the yield of primary MNs from each animal surgery was sufficient to measure neurite outgrowth, it was too limiting for the number of treatment and control groups needed for the signaling assays. This was further confounded by the highly variable and

unpredictable number of embryos from a given pregnant mouse, the intricacies of the surgery, and the culture conditions necessary to achieve the desired level of consistency in the results.

Given that BoNT/A effects on neurite outgrowth mimicked that of a neurotrophin, initial studies were also done to investigate the toxin's effect on cell survival using cultures of primary MNs. In this study, a LIVE/DEAD viability/cytotoxicity kit for mammalian cells from Molecular Probes was used to test whether BoNT/A increased the number of live primary MNs in culture. However, despite numerous repetitions, the data were inconsistent. Upon further investigation, it was determined that the inconsistencies were due to the variability in the number of dead cells since it was difficult to control how many dead cells detached and were washed off following each staining step. Additional experiments were done using another cell model, F11 cells. F11 is a hybrid cell line produced by fusion of neuroblastoma cells with dorsal root ganglion neurons (Fan et al., 1992). These experiments examined whether BoNT/A could promote F11 cell survival in the presence of hydrogen peroxide. Instead of the LIVE/DEAD assay, the standard thaizolyl blue tetrazolium bromide (MTT) assay was used for this study. A single experiment showed a slight but significant increase in cell survival in the toxin-treated group compared with both the untreated group and the hydrogen peroxide treated group; unfortunately this was not repeatable in other experiments (Fig 1.5). It was possible that toxin's protective effect was not strong enough to consistently prevent death caused by hydrogen peroxide, or the F11 hybrid cells have undetermined features that lead to significant variability. Regardless, the preliminary findings from both sets of initial experiments pointed to the need for a better, post-mitotic neuronal model to complete the dissertation studies.

For the majority of the studies presented in this dissertation, the HBG3 mESCs-derived MNs were chosen as the model preparation. These derived MNs are particularly good for neurite

outgrowth assays, since they express GFP and can be visualized with fluorescent microscopy without additional processing (Wichterle et al., 2002). Although human induced pluripotent stem cell (ipsc)-derived MNs are available and could be an ideal model, the differentiation process requires more delicate techniques and much longer culture periods compared to cells from mouse (Qu et al., 2014). It is important to note that HBG3-MNs were dissociated from embryoid bodies and grown in flat cultures, and these cultures consist of a mixed population of neural and glial cells. In preliminary work, the cultures were countered stained with DAPI to identify nuclei and an antibody against glial fibrillary acidic protein (GFAP) to distinguish glial cells. The results confirmed that the cultures consisted of around 40% MNs and 40% GFAP+ cells with the remaining 20% undetermined (Fig 1.6). It is likely that these GFAP+ cells could be astrocytes, although it is also possible that they could by neuronal precursor cells that give rise to neurons and oligodendrocytes (Menn et al., 2006). While it may be more desirable to have a purified MN culture (similar to primary cultures) for some of the studies in this dissertation, MNs mixed with glial cells in culture may be advantageous since glial cells support CNS neurons in vivo, making this model more representative of the in vivo environment (Azevedo et al., 2009). If necessary, future work can be done using fluorescence activated cell sorting to obtain purified GFP+ MNs for study. One disadvantage of these MNs is that when we tried to measure neurites using the high content analysis, the endogenously expressed GFP was not strong enough but we cannot enhance it like we did using fluorescent antibodies in other cells. As a result, we ended up using neurite outgrowth assay as introduced in the following chapters.

**Summary**

Neuritogenesis is a key process for neurons to establish their functional network both during development and after injury. It is regulated by different components, such as extracellular

factors, intracellular signaling pathways, transcription factors, and genes. Knowing the process during development might help us understand better the regeneration following injury. However, the mechanisms regulating different neuronal populations vary and how exactly MNs develop and regenerate is still unclear. BoNT/A has been shown to promote functional neuritogenesis in MNs. Although a few studies reported that some muscle-derived growth factors are involved in BoNT/A-induced neurite outgrowth, the molecular mechanisms underlying outgrowth that is independent of paralyzed muscle fibers are not known. To date, studies investigating critical cell signaling events involved in BoNT/A-induced neuritogenensis are non-existent. The current work will contribute greatly to our current understanding of BoNT/A-induced signaling transduction.

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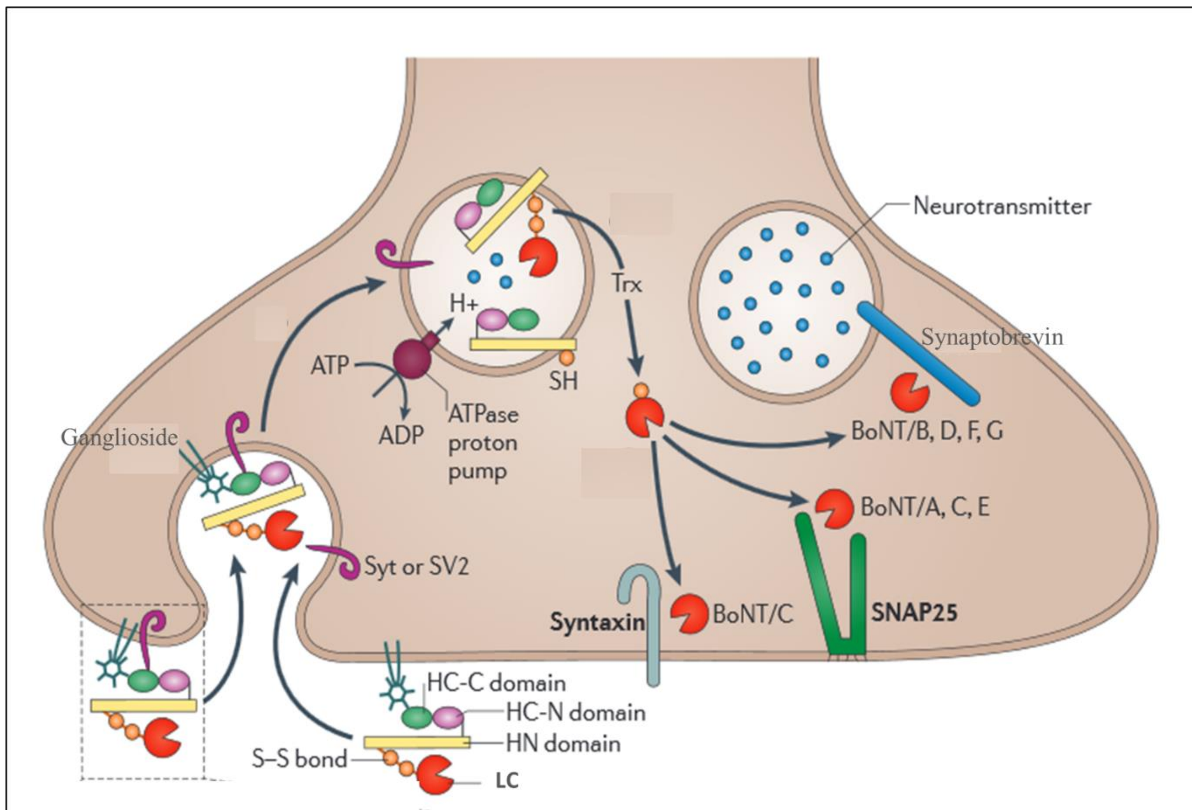


Figure 1.1: BoNT structure, receptors and targets. Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Microbiology] (Rossetto O, Pirazzini M and Montecucco C (2014) Botulinum neurotoxins: genetic, structural and mechanistic insights. *Nat Rev Microbiol* **12**:535-549.), copyright (2014).

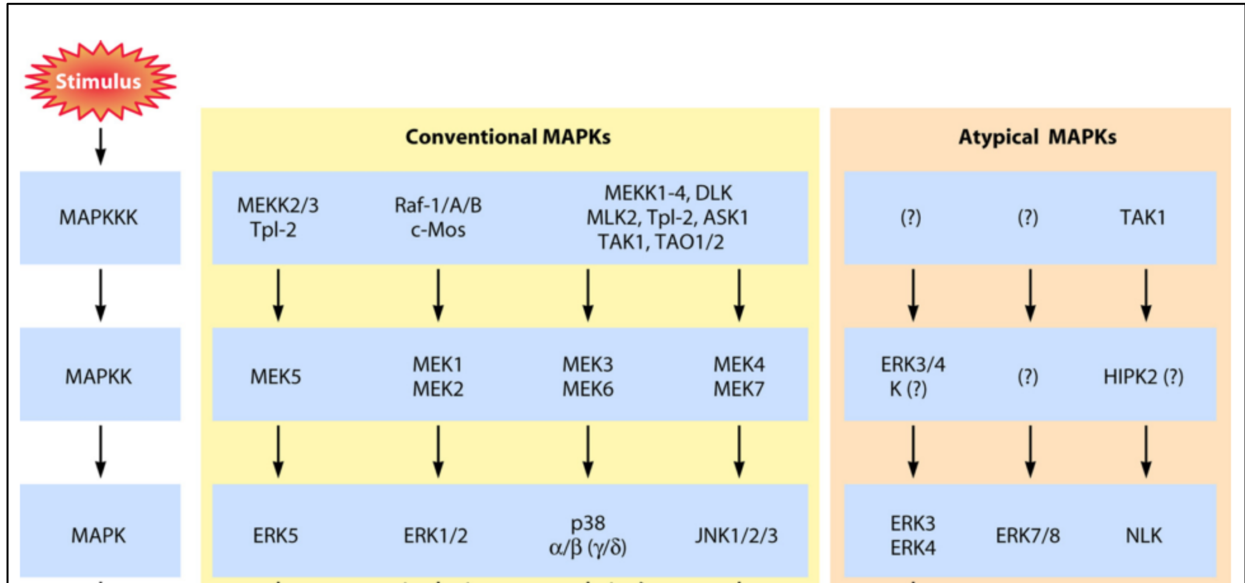


Figure 1.2: MAPK family. Adapted by permission from American Society for Microbiology: [Microbiology and Molecular Biology Reviews] (Cargnello M and Roux PP (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiology and molecular biology reviews : MMBR* **75**:50-83.), copyright (2011).

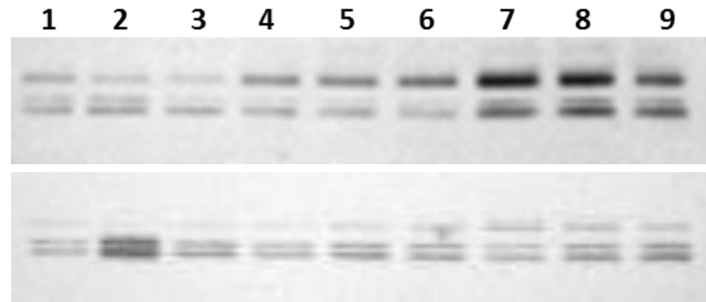


Figure 1.3: Detection of ERK phosphorylation after BoNT/A exposure in primary MNs. Protein samples were collected from mouse embryonic MNs, which had been cultured for 48 hours before treatment. Lane 1, control or no treatment; Lane 2, BoNT/A 1 nM for 10 minutes; Lane 3, GT1b 20 μM for 10 minutes; Lanes 4-9, BoNT/A 1 nM + GT1b 20 μM for 1, 2, 4, 6, 8 and 10 minutes, respectively. Upper and lower panels represent immunoblots probed with antibodies against p-ERK1/2 and pan-ERK1/2, respectively.

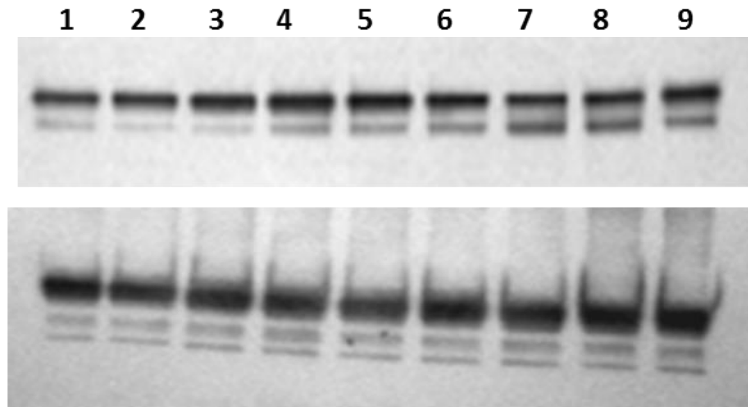


Figure 1.4: Detection of AKT phosphorylation after BoNT/A exposure in primary MNs. Protein samples were collected from mouse embryonic MNs, which had been cultured for 48 hours before treatment. Lane 1, control or no treatment; Lane 2, BoNT/A 1 nM for 10 minutes; Lane 3, GT1b 20 µM for 10 minutes; Lanes 4-9, BoNT/A 1 nM + GT1b 20 µM for 1, 2, 4, 6, 8 and 10 minutes, respectively. Upper and lower panels represent immunoblots probed with antibodies against p-AKT and pan-AKT, respectively.

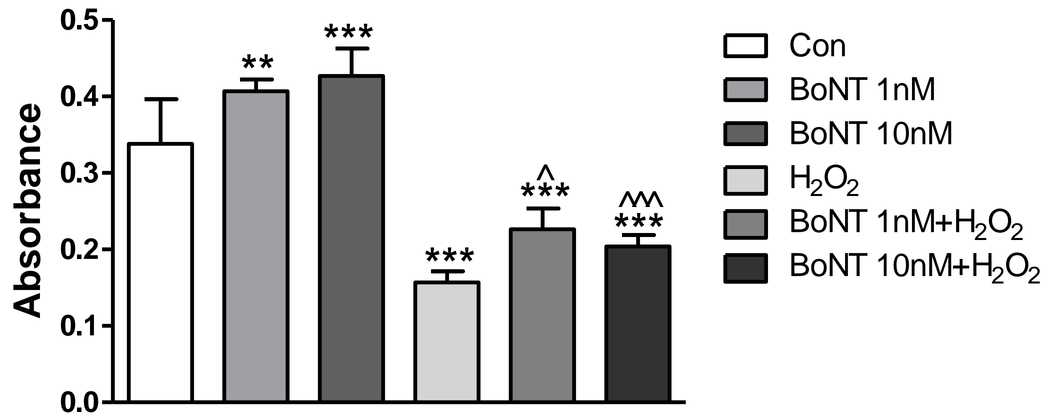


Figure 1.5: Neuroprotective effect of BoNT/A in F11 cells. F11 cells were treated with BoNT/A at 1 and 10 nM, $H_2O_2$ 30µM, or both for 3 hours with BoNT/A added 30 minutes ahead. MTT assay was used to determine the ratio of relative live cells compared to control group. $^{**}P<0.01$, $^{***}P<0.001$, compared with control group. $^{\wedge}P<0.05$, $^{\wedge\wedge\wedge}P<0.001$, compared with $H_2O_2$ only group.

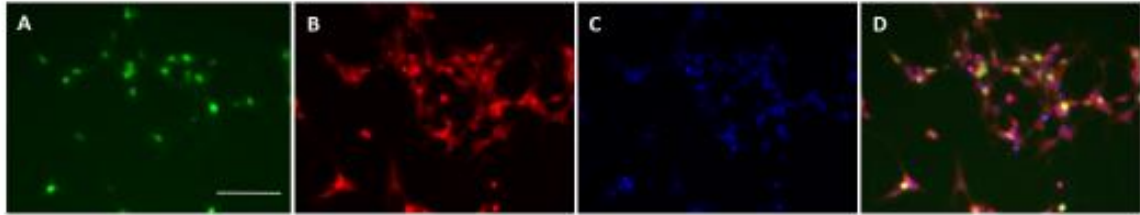


Figure 1.6: Characterization and quantification of cells in the HBG3 differentiated culture after 24 hours of seeding. A, GFP+ MNs; B, GFAP; C, DAPI; D, merged picture. Scale bar, 50 µm. Numbers of GFP+ and GFAP+ cells were counted using IPlab and compared with DAPI stained nucleus.

# CHAPTER 2

# ERK1/2 IS CRITICAL FOR NEURITOGENESIS IN DEVELOPING MOTOR NEURONS DERIVED FROM HBG3 STEM CELLS[1]

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**ABSTRACT**

Neurite development in motor neurons (MNs) is a fundamental process to establish neuronal/neuromuscular connectivity. It requires multiple steps of regulation, of which intracellular signaling transduction serves as a convergent point of both extrinsic and intrinsic factors. In this study, we aimed to determine if the extracellular signal-regulated kinases 1/2 (ERK1/2) pathway were involved in the regulation of neurite outgrowth in cultures of developing MNs. The HBG3 mouse embryonic stem cells (mESCs) derived MNs that express green fluorescent protein (GFP) were used as the in vitro model. Neurite outgrowth assays were first used to determine whether blocking the ERK1/2 pathway would suppress neurite growth and/or branching in cultures of the developing MNs. Results demonstrated that the pharmacological inhibitor U0126, which blocks ERK1/2 activation, reduced total neurite length and secondary branch formation, while primary neurite formation was not significantly altered. In addition, ERK1/2 phosphorylation was enhanced by bath applied brain-derived neurotrophic factor (BDNF), suggesting that BDNF-induced ERK1/2 activation could be one pathway mediating neurite development in cultures of HBG3-derived MNs. The AKT and classical protein kinase C pathways were also tested using the pathway-specific inhibitors LY294002 and GÖ6976 respectively. At the doses tested, LY294002 and GÖ6976 did not have significant effects on neurite development in culture. However, cytotoxicity was noted at high doses of LY294002. Taken together, these data indicate that the ERK1/2 pathway has a key role in mediating secondary neurite development in cultures of HBG3-derived MNs and could possibly be induced by BDNF.

**Key Words:** motor neuron, neurite outgrowth, ERK1/2, AKT, PKC, BDNF

## INTRODUCTION

Motor neurons diseases, such as amyotrophic lateral sclerosis and progressive muscular atrophy, are neurological disorders with motor neuron (MN) degeneration (Kanning et al., 2010). With the exception of the inheritable forms, little is known about the primary causes of these disorders, therefore effective treatments are lacking except for supportive management (McDermott and Shaw, 2008). While considerable research effort has been placed on developing new drugs, gene therapies or stem cell replacement, much less focus has been given to identifying the mechanism(s) underlying the MN degeneration and the associated disturbance in neurite connections. In this regard, an understanding of MN neurite development is fundamental and may provide clues to the causes of developmental/degenerative motor neuron diseases, which might further impact the treatment strategies.

Neurite outgrowth starts as early as neurons are committed (da Silva and Dotti, 2002). This usually requires multiple steps of regulation. For example, growth factors in the extracellular space first activate receptors on the neuronal membrane, followed by activation of intracellular signaling cascades, and finally changes in cell morphology through regulation of the cytoskeletal system or gene transcription. The intracellular signaling pathways serve as a convergence of both extrinsic and intrinsic factors, although this varies across the extracellular environment and cell type (Khodosevich and Monyer, 2010; Sainath and Gallo, 2015). Pathways mediating neuritogenesis are numerous and include, but are not limited to, mitogen-activated protein kinases (MAPK), protein kinase B (PKB; aka AKT), protein kinase C (PKC), protein kinase A (PKA), and the Rho GTPases (Rho/Rac1/Cdc42) (Duman and Voleti, 2012; Sainath and Gallo, 2015). Although some studies have reported changes in signaling pathways under specific conditions, the exact

mechanisms for neuritogenesis of MNs are largely unknown (Ozdinler and Macklis, 2006; Aglah et al., 2008; Soundararajan et al., 2010; Newbern et al., 2011).

In this study, we use an in vitro model of developing MNs to investigate the role of the extracellular signal-regulated protein kinase 1/2 (ERK1/2, a member of the MAPK family), AKT and PKC pathways in normal MN neurite development. MNs derived from HBG3 mouse embryonic stem cells (mESCs) were used as the in vitro model. HGB3 derived MNs (HBG3-MNs) possess a *GFP* gene introduced into the promoter region of the *Hb9* gene, the latter of which serves as a postmitotic marker of MNs (Wichterle et al., 2002); therefore, when the mESCs differentiate into MNs, they spontaneously express strong green fluorescence. Neurite outgrowth assays using specific signaling pathway inhibitors showed that ERK1/2 kinases played a critical role in regulating neurite length and secondary branch formation in these neurons. The ERK1/2 pathway has been reported to be a downstream effector of neurotrophins like brain-derived neurotrophic factor (BDNF) (Cohen-Cory et al., 2010). BDNF has been reported to have direct protective effects on MNs (Kishino et al., 1997; Naeem et al., 2002). Not surprisingly, applying exogenous BDNF in our culture system significantly enhanced ERK1/2 activation in HBG3-MNs. On the other hand, pharmacologic inhibitors of the AKT and classical PKC pathways at the doses tested did not block neuritogenesis significantly. Interestingly, none of the three pathways examined appear to play a role in the formation and growth of primary neurites, suggesting differential regulation of primary and secondary neurite outgrowth.

## MATERIALS AND METHODS

**Cell Culture.** The HBG3 mESCs (a kind gift from Dr. Steven L. Stice, University of Georgia, Athens, GA) were maintained on a layer of Mitomycin C inactivated mouse embryonic fibroblasts (MEFs) in DMEM media (EMD Millipore, Billerica, MA) supplemented with 15% defined fetal bovine serum (Hyclone, Fisher Scientific, Pittsburgh, PA), 100 U/ml leukemia inhibitory factor (LIF, EMD Millipore), 2 mM L-glutamine with penicillin/streptomycin (Gibco, Life Technologies, Grand Island, NY), 1% non-essential amino acids (EMD Millipore), 1% nucleosides (EMD Millipore) and 0.1 mM β-mercaptoethanol (Gibco). The HBG3 cells were kept under 15 passages for the following study.

Motor neurons were differentiated as described previously (Mazzoni et al., 2011). Briefly, at day 0 (D0), HBG3 cells were cultured in the ADFNK differentiation media which is 1:1 mixture of Advanced DMEM/F12 medium (Gibco) with Neurobasal medium (Gibco), 10% Knockout serum replacement (Gibco), 2 mM L-glutamine with penicillin/streptomycin and 0.1 mM β-mercaptoethanol. At D2, the differentiation medium was supplemented with 1 µM retinoic acid (RA, Sigma-Aldrich, St. Louis, MO) and 100 ng/ml sonic hedgehog (Shh, R&D Systems, Minneapolis, MN). At D5, the differentiation medium was further supplemented with 10 ng/ml glial-derived neurotrophic factor (GDNF, Biosource International, Camarillo, CA Biosource) and 2 ng/ml ciliary neurotrophic factor (CNTF, Biosource International), in the presence of RA and Shh. At D6, embryoid bodies were dissociated with 0.05% trypsin-EDTA (Gibco) for 4 minutes at 37˚C and plated in 24-well plates coated with matrigel (BD Biosciences, San Jose, CA).

**Neurite Outgrowth Assay.** Cells were plated at a density of 33,000 cells per well and maintained in ADFNK media supplemented with 10 ng/ml GDNF, 2 ng/ml CNTF, 2% B27 (Gibco) and 1% N2 (Gibco). The cultures were kept in a humidified incubator for 30 minutes to ensure attachment before further treatments.

After 30 minutes of attachment, media were supplemented with one of the pathway inhibitors (U0126 (Cell Signaling Technology, Danvers, MA), LY294002 (Cell Signaling Technology), GÖ6976 (EMD Millipore) for 6 or 24 hours.

GFP-positive MNs were then visualized using an inverted epi-fluorescent microscope and images were captured. Culture plates were viewed in a grid pattern, and cells meeting the following criteria were captured using MagnaFIRE SP software: 1) cells showing strong GFP intensity; 2) cells extending at least one neurite with the length of the longest neurite not less than two times the soma diameter; and 3) somata displaying a pyramidal like morphology. Cells that were rounded up and not extending neurites were not included since this may be indicative of cell detachment and poor health. Cellular morphometrics were analyzed using IPLab software. Total neurite length was measured; protrusions less than one soma diameter were excluded. Primary and secondary branches were counted. One-way ANOVA analysis with Dunnetts' post hoc tests were used for statistical significance (GraphPad Software, San Diego, CA).

**Western Blot Assay.** After 30 minutes for seeding, cultures were pre-incubated with 10 nM U0126 for 30 minutes, and then supplemented with 50 ng/ml BDNF (Biosource International) for up to 1 hour. Cellular contents were harvested using T-Per protein extraction reagent with protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA), followed by brief sonication and centrifugation. Protein samples (30 µg/lane) were resolved on 4-20% Tris-HCL gels and transferred to polyvinylidene difluoride membrane. Monoclonal antibodies to p-ERK1/2 (1:1000, Cell Signaling Technology), pan-ERK1/2 (1:1000, Cell Signaling Technology) were used to probe for the cognate protein employing a standard western immunoblot protocol. Immunoreactivity was visualized with chemiluminescence and the results were analyzed using Quantity One (BioRad, Hercules, CA).

**Thaizolyl blue tetrazolium bromide (MTT) Assay**. Cells were seeded in matrigel coated 96-well plates at the density of 33,000 cells per well. Cells were allowed to attach for 30 minutes, then incubated in LY294002 at the final concentrations ranging from 10 to 50 μM. Dimethyl sulfoxide (DMSO, 0.5%, Sigma-Aldrich) was used as a vehicle control. After 24 hours of incubation, 0.5 mg/ml MTT (Sigma-Aldrich) was added into each well and cultured for another 4 hours before removing media. DMSO 100 μl/well was used to dissolve crystals. Absorption was measured at 570 nm.

## RESULTS

### MEK1/2 Inhibitor U0126 Blocks Neurite Outgrowth in HBG3-MNs

Following differentiation into MNs, the cultured HBG3 cells spontaneously displayed green fluorescence that was detected by standard fluorescence microscopy. The individual MNs grew well on matrigel-coated plates and extended neurites, with morphology similar to primary embryonic MNs but a faster growth rate, indicating that they are good candidates for neurite outgrowth assays (Fig 2.1A). To assess the role of the ERK1/2 pathway in neurite outgrowth, the kinase inhibitor U0126 was added to culture media 30 minutes after cells were plated and neurites were assessed at 6 and 24 hours post-application. The inhibitor blocks the activation of mitogen-activated protein kinase kinase 1/2 (MEK1/2) (Cargnello and Roux, 2011). MEK1/2 is the directly upstream of ERK1/2 and activates ERK1/2 via phosphorylation. U0126 interacts with inactive MEK1/2 strongly to prevent its activation.

Commonly studied morphometric features of neurite outgrowth include neurite length, neurite number and neurite branching. Figure 2.1(B-D) illustrates the effects of 10, 30 and 50 μM

U0126 on neurite outgrowth measured at 6 and 24 hours after application. Compared to time-matched, vehicle treated controls, total neurite length was significantly reduced in the presence of U0126 (Fig 2.1B). At 6 hours, total length decreased by 16.8% at 10 µM, 24.9% at 30 µM, and 24.2% at 50 µM. While a greater effect was noted at the higher doses, no significant difference between dose groups was evident. Similar results were observed at 24 hours: with total length reduced by 23.5%, 28.7% and 40.4% at each dose respectively. In addition to neurite length, the number of primary and secondary branches were also measured (Fig 2.1C and 2.1D). As the U1026 dose increased, the number of secondary neurites decreased by 28.6%, 30.1% and 30.1% at 6 hours, and 26.4%, 30.3%, and 35.6% at 24 h. Interestingly, U1026 had no measurable effect on primary branch numbers at any of the three doses, at either time point.

**ERK1/2 Phosphorylation is Enhanced by BDNF**

BDNF has been shown to promote axon/dendrite outgrowth and can be synthesized in MNs (Jeong et al., 2011; Joseph et al., 2012). Exogenous BDNF promoted neurite length and secondary neurite formation in HBG3-MNs (data presented in chapter 4 of this dissertation). To examine the role of the ERK1/2 pathway in BDNF action, exogenous BDNF was bath applied to the cultures and ERK1/2 phosphorylation was measured. BDNF increased ERK1/2 phosphorylation by 114.9% after 30 minutes of incubation, and such enhancement of p-ERK1/2 returned to baseline by 1 hour (Fig 2.2). These data indicated that BDNF induces transient ERK1/2 pathway activation to mediate the cell response. When MNs were pretreated with U0126, no phosphorylated ERK1/2 was detected, either in the absence or presence of BDNF. Collectively, these data suggests that the ERK1/2 pathway is activated by BDNF, and may potentially mediate neurite outgrowth induced by BDNF.

**Inhibition of the AKT Pathway Induces Cytotoxicity but Has no Effect on Neurite Outgrowth in MNs**

The AKT pathway plays a critical role in neuronal survival and has emerged as a permissive mediator of neurite outgrowth (Namikawa et al., 2000). A gain of function of the AKT pathway has been reported to promote axon regeneration in a nerve injury model (Namikawa et al., 2000). However, the effects of loss of AKT function by pharmacologic inhibition has not been conclusive (Lim and Walikonis, 2008; Wang et al., 2011). Since phosphoinositide 3-kinase (PI3K) activates the AKT pathway, we tested the role of AKT pathway in HBG3-MN neuritogenesis using the PI3K inhibitor LY294002. Three dose groups ranging from 10 µM to 50 µM were tested at 6 and 24 hours and compared to time-matched, vehicle-treated controls. LY294002 at 10 µM had no cytotoxic effect (Fig 2.3), and showed no effect on total neurite length, numbers of primary or secondary neurites in HBG3-MNs (Fig 2.4). Cultures treated with higher doses of LY294002 showed significant cytotoxicity (Fig 2.3). However, in cultures demonstrating significant cytotoxicity the remaining live cells had no difference in neurite outgrowth compared to vehicle-treated controls at either time point (Fig 2.4).

**Blocking PKC Does Not Alter the Development of Neurites in MNs**

Preliminary data (see chapter 4) indicated that PKCα mRNA was up-regulated upon stimulation, therefore it was of interest to examine whether PKCα is involved in development of neurites of MNs. The PKC inhibitor GÖ6976 was used to study the involvement of the PKC pathway in neuritogenesis. Three doses of the inhibitor were chosen to selectively block PKCα (2.3nM), PKCβI (6.2nM) or non-specific PKC (7.9nM). None of these doses had any effect on neurite length (Fig 2.5) or branching (data not shown) in HBG3-MNs.

## DISCUSSION

Neurite outgrowth is a critical process for neurons to establish intercellular networks during differentiation, migration and regeneration (Thiede-Stan and Schwab, 2015). It is an integrated response from attractive/repulsive and long-range/short-range cues in the environment. Although signaling pathways mediating neuritogenesis have been studied for decades, results vary significantly across cell type, extracellular positive or negative stimuli, growing conditions of cells, and other variables. For example, neurite elongation in hippocampal neurons induced by hepatocyte growth factor was mediated by the AKT pathway, while the same phenomenon required both the AKT and MAPK pathways when stimulated by fibroblast growth factor (FGF) (Lim and Walikonis, 2008; Vantaggiato et al., 2011). Conversely, in neurons derived from adult rat spinal cord progenitor cells, the AKT pathway was not involved in neurite outgrowth (Chan et al., 2013). To date, the research on signaling cascades from the limited number of studies done in MNs shows variant results (Harper et al., 2004; Ozdinler and Macklis, 2006; Aglah et al., 2008; Soundararajan et al., 2010). Based on this, the current study focused on the signaling transduction pathways inside MNs with limited environmental inducers or inhibitors, aiming to provide better understanding of the development of MNs and provide potential therapeutic hints for motor neuron diseases.

A number of findings indicate that HBG3-MNs possess characteristics that make them good candidates for MN related research. HBG3-MNs are spontaneously GFP positive which allows researchers to visualize them both in vivo and in vitro easily (Wichterle et al., 2002; Miles et al., 2004). This is due to the introduction of an enhanced *GFP* gene into embryonic stem cells that becomes expressed under the control of the *Hb9* gene promoter. The product of *Hb9* is a homeodomain transcription factor that is expressed in mice beginning from ED9.5 and is a marker

of postmitotic MNs. Besides *Hb9*, these derived cells also express other characteristics such as choline acetyltransferase, SNAP-25, and synaptic vesicle glycoprotein 2A which are necessary components of a functional cholinergic nerve terminal (Kiris et al., 2011). Additionally, Wichterle et al. demonstrated that the HBG3-MNs can populate into the spinal cord and innervate target muscles (Wichterle et al., 2002). In the current study, the HBG3-MNs expressed similar morphological attributes to primary MNs previously studied in this laboratory, confirming their potential for use in neurite analyses (Coffield and Yan, 2009).

The ERK1/2 pathway, a member of the MAPK family, is known to have key functions in cell proliferation, as well as significant roles in differentiation, migration, and survival (Ebisuya et al., 2005; Cargnello and Roux, 2011). Not only do ERK1/2 kinases trigger various biological responses, their activation sometimes leads to opposite outcomes of the same cellular processes (e.g. apoptosis vs survival), mostly depending on the duration, strength or subcellular localization of ERK activation. Current literature on the requirement of ERK1/2 in MNs is limited and with inconsistent results. For example, one study suggested that in MNs the ERK1/2 pathway mediated neurite extension and axon guidance when induced by FGF; whereas, a different study using forskolin reported that neurite outgrowth was regulated independently of ERK1/2 pathway, when the cAMP-PKA pathway is invoked (Aglah et al., 2008; Soundararajan et al., 2010). This discrepancy brings up the question of whether the ERK1/2 pathway is only elicited by specific growth factor/environmental stimulants, or whether this pathway is required in the basal development of neurites and further enhanced by certain growth factors. In the current study, MNs were cultured in media with only GDNF and CNTF neurotrophins, which are used as standards in many protocols for MN culture (Soundararajan et al., 2006; Kiris et al., 2011). Both GDNF and CNTF have been shown to promote survival of MNs (Lamas et al., 2014). The results of the current

studies in which the MEK1/2 inhibitor U0126 was applied to developing neurons indicate that the ERK1/2 pathway mainly regulates secondary neurite formation and total neurite length. In addition, reduction of total length by 50 µM U0126 at 24 hours was significantly different from 10 µM groups, suggesting that some other pathways which were blocked by high dose of U0126 might be involved in neurite extension over time. ERK5, another member in the MAPK family, could be one possibility. ERK5 has been reported to regulate neurite outgrowth in PC12 cells and was blocked by U0126 at 50 µM (Esparis-Ogando et al., 2002; Obara et al., 2009). There are few studies done in MNs and more studies are needed to test this hypothesis. Interestingly, the number of primary neurites was not reduced by U0126. Similarly, a study by Newbern, et al using $ERK1/2^{CKO}$ showed that ERK1/2 knockout did not affect MN axon innervation of muscles (Newbern et al., 2011); together these findings suggest the involvement of other pathways to regulate primary branch formation.

After revealing the critical role of ERK1/2 in the basal development of HBG3-MN secondary neurites, we further confirmed that the ERK1/2 pathway can be a direct downstream effector of BDNF signaling in these MNs. BDNF is a member in the NTF family and is crucial for the development and function of neurons (Connor and Dragunow, 1998; Saha et al., 2006). It works locally, either secreted by postsynaptic targets (e.g. neurons, muscle fibers and astrocytes) or via an autocrine mechanism. During embryogenesis, BDNF supports neural tube development and neuronal precursor cell differentiation. Later in the mature nervous system, it regulates neuronal branching and remodeling, as well as synaptic plasticity (Kernie et al., 2000). In MNs, BDNF up-regulation is associated with improvement of motor function or motor neuron regeneration (Kishino et al., 1997; Naeem et al., 2002). Efforts to enhance BDNF levels are one of the direct ways to promote the recovery of motor neuron injury or disease (Ying et al., 2005;

Yamauchi et al., 2006; Boyce et al., 2007). Our findings suggest that BDNF activated the ERK1/2 pathway to stimulate neurite development in HBG3-MNs, which is consistent with studies in other neuronal cell types (Alonso et al., 2004).

The AKT pathway plays a critical role in neuronal survival and emerges as a positive mediator of neurite outgrowth (Read and Gorman, 2009). The permissive function in neuritogenesis has been reported in many neuronal cell types including hippocampal neurons, cerebellar neurons, and cochlear spiral ganglion neurons (Lim and Walikonis, 2008; Okada et al., 2011; Mullen et al., 2012). Although overexpression of AKT has been associated with axon regeneration in injured adult MNs, it is difficult to separate survival from neuritogenesis in this case, since enhanced neurite outgrowth might be a consequence of promoting cell survival (Namikawa et al., 2000). Using a pharmacological inhibitor to block AKT activation in the current study and then observing changes in survival and neurite outgrowth may help to answer this question. Not surprisingly, high doses of the AKT inhibitor did result in significant cytotoxicity in treated cultures, whereas neurite outgrowth in the MNs was not impeded at any dose of inhibitor, although less GFP+ neurons were present in groups with high doses of AKT inhibitor. These data suggest that the AKT pathway does not directly regulate neurite outgrowth in these MNs. However it is important to note that while 10 μM LY294002 has been reported to inhibit AKT phosphorylation in neurons by others, phosphorylation of AKT in the presence of LY294002 was not examined in the current study. Thus, further studies are needed to confirm the findings reported herein (Endo et al., 2006; Guo et al., 2011; Cheng et al., 2013).

PKC affects many cellular responses including neuronal migration and neurite outgrowth through the regulation of the cytoskeletal microfilaments (Larsson, 2006). PKC isozymes are grouped into classical (α, βI, βII and γ), novel (δ, ε, η and θ) and atypical PKCs (ι/λ and ξ) (Bright

and Mochly-Rosen, 2005). Most of them have expressions in the brain and spinal cord. Of the three groups, novel PKC isozymes have been reported most frequently to promote neurite outgrowth in multiple cell types (Larsson, 2006). Classical PKCs, blocked by the inhibitor GÖ6976 specifically, seem less involved (Troller et al., 2001). Since our preliminary PCR microarray analysis indicates the up-regulation of PKCα mRNA upon stimulation by botulinum neurotoxin, it was of interest to ask if PKCα is involved in neurite outgrowth of MNs. Our results are consistent with current literature on the classical PKCs, showing no marked blockade of neurite development in MNs when GÖ6976 was applied at the relevant doses, suggesting that this up-regulation functions beyond neuritogenesis. In addition, although crosstalk between the PKC and ERK1/2 pathways has been reported, our data suggest that classical PKCs do not influence ERK1/2-mediated neurite outgrowth in these MNs (Wu et al., 2012). Although the doses for GÖ6976 used in the current study have been tested by the manufacturer and reported by others to block PKC in several cell types, many published research in neurons tested much higher doses (0.1 to 2 μM), suggesting that a dose response experiment covering a wider range in the HBG3-MNs cultures will be necessary to confirm the findings of the current study (Zhang et al., 2006; Wang et al., 2014; Higa-Nakamine et al., 2015; Xu et al., 2015). In addition, examining the phosphorylation of PKC as a measure of activation would strengthen the current findings (Zhang et al., 2006; Zhou et al., 2006). The activation of PKC involves two steps that contribute equally (Newton, 1995). First, PKC needs to be phosphorylated and then substrate comes in to remove the pseudosubstrate from the binding site. Activated PKC further phosphorylates its substrates to exert a cellular response. Comparing the degree of substrate phosphorylation is another way to measure PKC activity (Koivunen et al., 2004; Zhou et al., 2006). More work needs to be done to elucidate roles of PKCs on HBG3-MN neuritogenesis.

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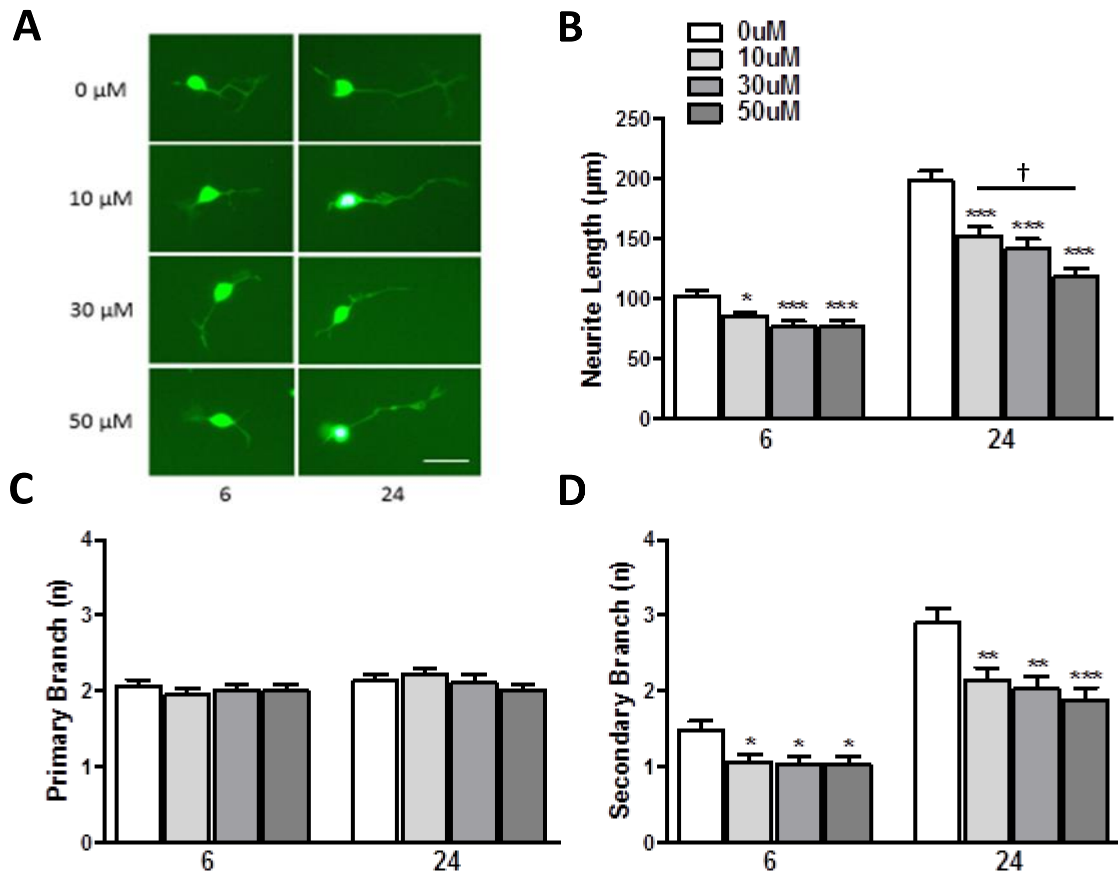


Figure 2.1: Effects of U0126 on neurite outgrowth in cultures. A, fluorescent images of MNs from cultures treated with bath applied U0126 from 0 to 50 µM at 6 and 24 hours. Scale bar, 50 µm. B-D, comparison of total neurite length, number of primary and secondary neurites treated with increasing concentrations of U0126 at 6 and 24 hours (mean ± S.E.M., n=90). *$P$<0.05, **$P$<0.01, ***$P$<0.001, compared with 0 µM U0126 at each time point. †$P$<0.05, comparison of neurite length between 10 and 50 µM at 24 hours.

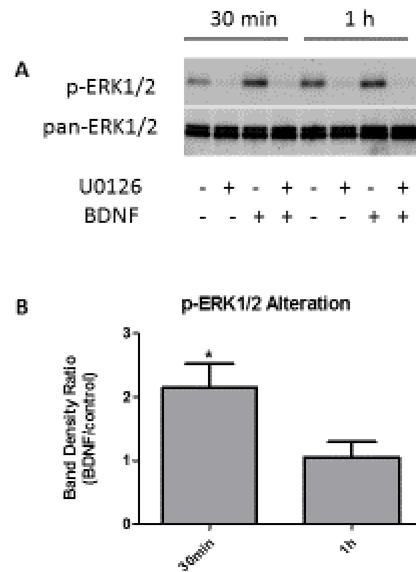


Figure 2.2: Effect of BDNF treatment of HBG3-MN cultures on ERK1/2 phosphorylation. A, Immunoblot of p-ERK1/2 and pan-ERK1/2 after 10 μM U0126, or 50 ng/ml BDNF, or both for 30 minutes and 1 hour. B, comparison of relative band density ratio (p-ERK/pan-ERK) between BDNF and no treatment group at each time point. *$P<0.05$.

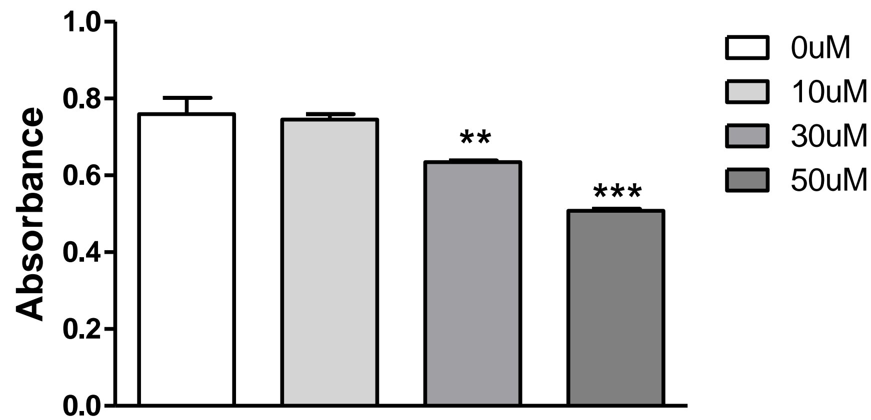


Figure 2.3: Cell toxicity treated by LY294002 in cultures of HBG3-MNs. MTT staining was used to quantify cell viability after exposing cells to LY294002 at increasing concentrations for 24 hour (mean ± S.E.M, n=6). ***P*<0.01, ****P*<0.001, compared with 0 μM concentration.

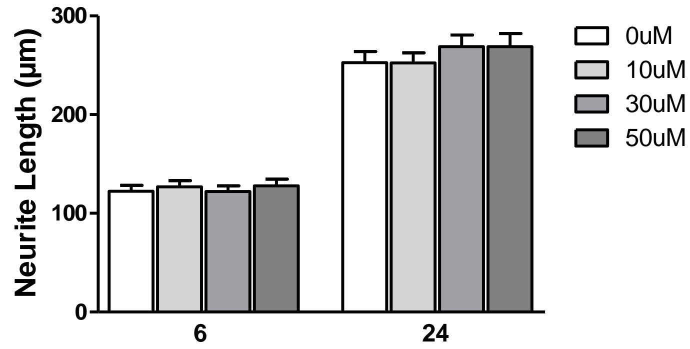


Figure 2.4: Effects of LY294002 treatment on neurite length. HBG3-MNs were treated with LY294002 from 0 to 50 µM. Neurite length were compared with 0 µM at both 6 and 24 hours (mean ± S.E.M, n=90).

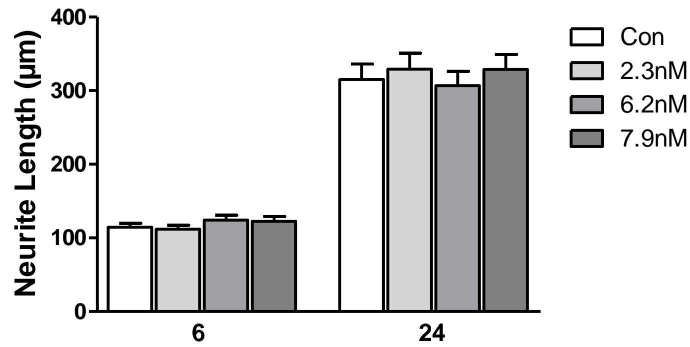


Figure 2.5: Effect of GÖ6976 treatment on neurite length. HBG3-MNs were treated with GÖ6976 at 2.3 nM (PKCα), 6.2 nM (PKCβI) and 7.9 nM (PKC in general). Neurite length were compared with control at both 6 and 24 hours (mean ± S.E.M, n=90).

# CHAPTER 3

# INHIBITION OF ERK1/2 SIGNALING ATTENUATES BOTULINUM NEUROTOXIN-INDUCED NEURITE OUTGROWTH IN STEM CELL-DERIVED MOTOR NEURONS[1]

[1]Liyun Liu and Julie Coffield. To be submitted to *Pharmacology and Experimental Therapeutics.*

**ABSTRACT**

Botulinum neurotoxin serotype A (BoNT/A), known to produce neuromuscular paralysis (botulism) with extremely high potency is also a well-established therapeutic agent used to alleviate the pain and dysfunction associated with a number of neurologic and muscular disorders. Independent of the cellular mechanism that causes botulism, BoNT/A has been found to promote neurite outgrowth of motor neurons. This phenomenon, sometimes referred to as 'sprouting', may be beneficial by initiating recovery from botulism, but may be less desirable by diminishing BoNT/A's therapeutic effect over time. The cellular mechanism(s) underlying this phenomenon remain unknown. In the current study motor neurons differentiated from mouse stem cells (HBG3-MNs) were used to investigate potential signaling pathways regulating BoNT/A-induced neurite outgrowth. BoNT/A-stimulated outgrowth in HBG3-MNs was similar to our previously published findings for mouse primary MNs. Our findings revealed that phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2), a marker of ERK1/2 activation, was enhanced transiently in response to BoNT/A in the culture. Neurite outgrowth assays using pharmacologic inhibitors [(U0126, mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor; LY294002, phosphoinositide 3-kinase (PI3K) inhibitor; and GÖ6976, protein kinase C (PKC) inhibitor] demonstrated that U0126, which blocks ERK1/2 activation, reduced the stimulatory effects of BoNT/A on primary and secondary branch formation as well as total neurite length in HBG3-MNs. Neither LY294002 nor GÖ6976 inhibited BoNT/A-induced outgrowth at the doses tested, and U0126 had no effect on the toxin's well recognized ability to cleave SNAP-25, the mechanism known to produce paralysis. Collectively, these data suggest that ERK1/2 signaling may play a critical role in BoNT/A-induced neuritogenesis and sprouting.

**Key Words:** Botulinum Serotype A, Neuritogenesis, HBG3-MNs, U0126, ERK1/2 phosphorylation, Sprouting

## INTRODUCTION

Botulinum neurotoxin (BoNT), the most potent biological toxin present in nature, is classified as a Tier 1 select agent, and as such "presents the greatest risk of deliberate misuse with most significant potential for mass casualties or devastating effects to the economy, critical infrastructure, or public confidence" (Centers for Disease et al., 2012). BoNT selectively targets peripheral synapses, particularly those found at the neuromuscular junction, with extreme potency and causes botulism (Simpson, 2004; Sobel, 2005; Smith, 2009). The typical clinical manifestation of botulism is a symmetrical, flaccid paralysis that starts in the face and descends towards the limbs, which may be lethal if not treated. Although the disease may eventually resolve with intensive supportive care, significant time (3-6 months) is required for recovery of synaptic function and neurotransmission at the affected nerve terminals. The molecular mechanism underlying botulism is the potential for BoNT, a metalloprotease, to interfere with the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins that mediate vesicle-dependent exocytosis of neurotransmitter. There are seven known serotypes of BoNT (A-G) plus one recently proposed serotype H (Dover et al., 2014). Type A (BoNT/A) selectively cleaves the SNARE protein synaptosome-associated protein 25 (SNAP-25), between residues $Gln^{197}$ to $Arg^{198}$ (Binz et al., 1994). Despite its recognized lethality and potential for misuse, BoNT has gained considerable popularity as a therapeutic agent for an ever-expanding list of conditions. Localized injections of primarily BoNT/A may be used in clinical practice to treat a variety of

disorders of dystonia, spasticity, or headache and BoNT/A is routinely used to minimize facial wrinkles (Vaidyanathan et al., 1999; Jankovic, 2004).

Interestingly, BoNT/A has been shown to stimulate axon sprouting at paralyzed neuromuscular junctions (de Paiva et al., 1999). This phenomenon is thought to contribute to the onset of recovery following paralysis. In addition, it has been demonstrated that BoNT/A promotes neurite outgrowth in cultured primary motor neurons (MNs) (Coffield and Yan, 2009). The underlying cellular mechanism is unclear and independent of that which produces paralysis. A few studies indicate that muscle fiber-derived extracellular molecules, such as insulin growth factor (IGF), might partially mediate the sprouting observed in BoNT-paralyzed muscles; however, the intrinsic neuronal factors involved in BoNT-induced neurite outgrowth have not been studied (Caroni and Schneider, 1994; Caroni et al., 1994).

Potential signaling pathways mediating neuritogenesis are numerous and include, but are not limited to, protein kinase A, PI3K-AKT (aka protein kinase B), protein kinase C (PKC), mitogen-activated protein kinases (MAPK), and Rho GTPases (Buchser et al., 2010). ERK1/2 is an important signaling kinase that belongs to the MAPK family (Roskoski, 2012). Neurotrophins, critical regulators of neuritogenesis, bind to membrane tropomyosin-related kinase (TRK) receptors and trigger receptor dimerization and auto-phosphorylation, leading to a cascade of intracellular events (Huang and Reichardt, 2003). During development, neurotrophins often invoke AKT, PKC and/or ERK1/2 pathways (Segal, 2003).

Commonly studied morphometric aspects of neuritogenesis include neurite length, neurite number and neurite branching. In the current study, we examined the function of the ERK1/2 (MAPK), AKT and PKC pathways in BoNT/A-induced neurite outgrowth using cultured HBG3-MNs derived from mouse embryonic stem cells (mESC). The advantage of the HBG3 mESC line

in particular is that an *eGfp* gene has been introduced into the promoter region of the *Hb9* gene, which is a known post-mitotic marker of MNs (Wichterle et al., 2002). Following differentiation to MNs, the cultured cells will spontaneously display green fluorescence that can be detected by standard fluorescence microscopy. Previous work reported that these cells are sensitive to BoNT/A (Kiris et al., 2011). As reported here, our findings indicate that treatment of HBG3-MNs with BoNT/A stimulated neurite outgrowth in a manner similar to what has been reported previously using mouse primary spinal MNs (Coffield and Yan, 2009). This stimulation of neurite outgrowth was accompanied by a transient elevation in the phosphorylation of ERK1/2 protein in response to BoNT/A treatment, indicating ERK1/2 activation in the culture. Furthermore, in the presence of the MEK1/2 inhibitor U0126, which prevents the activation of ERK1/2, BoNT/A-induced changes in neurite elongation and branching were blocked. Conversely, AKT and PKC pathway inhibitors did not alter toxin-induced neurite outgrowth. Inhibition of ERK1/2 activation had no effect on BoNT/A-induced cleavage of SNAP-25. Collectively, these findings reveal that ERK1/2 signaling is a critical intracellular mediator of BoNT/A-induced neurite outgrowth.

## MATERIALS AND METHODS

**Cell Culture.** The HBG3 cells were cultured on a layer of Mitomycin C-inactivated mouse embryonic fibroblasts (MEF) in DMEM (EMD Millipore, Billerica, MA) with 15% defined FBS (Hyclone, Fisher Scientific, Pittsburgh, PA), 2 mM L-glutamine with penicillin/streptomycin (Gibco, Grand Island, NY), 1% non-essential amino acids (EMD Millipore, Billerica, MA), 1% nucleosides (EMD Millipore), 100 U/ml leukemia inhibitory factor (LIF, EMD Millipore) and 0.1 mM β-mercaptoethanol (Gibco).

Motor neuron differentiation was carried out as described previously (Mazzoni et al., 2011). Beginning at day 0 (D0), mESC were cultured in ADFNK medium, which is a 1:1 mixture of Advanced DMEM/F12 medium (Gibco) with Neurobasal medium (Gibco), supplemented with 10% Knockout serum replacement (Gibco), 2 mM L-glutamine with penicillin/streptomycin and 0.1 mM β-mercaptoethanol. After 2 days in aggregate culture to form embryoid bodies (EBs), the ADFNK medium was supplemented with 1 μM retinoic acid (RA, Sigma-Aldrich, St. Louis, MO) and 100 ng/ml sonic hedgehog (Shh, R&D Systems, Minneapolis, MN). At D5, the medium was further supplemented with 10 ng/ml glial-derived neurotrophic factor (GDNF, Biosource International, Camarillo, CA) and 2 ng/ml ciliary neurotrophic factor (CNTF, Biosource International), in the presence of RA and Shh. At D6, EBs were dissociated with 0.05% trypsin-EDTA (Gibco) for 4 minutes at 37˚C. Dissociated cells were then seeded in 24-well plates coated with matrigel (BD Biosciences, San Jose, CA). After 6 hours of culture, GFP-positive MNs constituted approximately 40% of the total population.

**Toxin Treatment.** Cells were cultured in ADFNK medium supplemented with 10 ng/ml GDNF, 2 ng/ml CNTF, 2% B27 (Gibco) and 1% N2 (Gibco). The cultures were maintained in a humidified incubator for 30 minutes to ensure cell attachment. Following cell attachment, the culture medium was supplemented with either pure BoNT/A (Metabiologics, Madison, WI), BoNT/A plus one of the pathway inhibitors U0126 (Cell Signaling Technology, Danvers, MA), LY294002 (Cell Signaling Technology) or GÖ6976 (EMD Millipore), or the inhibitor alone. In the toxin plus inhibitor experiments, BoNT/A was added 30 minutes following addition of the inhibitor. Each experimental paradigm was replicated three times.

**Neurite Outgrowth Assay**. GFP-positive neurons were visualized using an inverted epi-fluorescent microscope and images were captured 6 and 24 hours after the addition of toxin.

Culture wells were viewed in a grid pattern, and cells meeting the following criteria were captured using MagnaFIRE SP software: 1) cells showing strong GFP intensity; 2) cells extending at least one neurite with the length of the longest neurite not less than two times the soma diameter; and 3) somata displaying a pyramidal like morphology. Cells that were rounded up and not extending neurites were not included since this may be indicative of cell detachment and poor health. Cellular morphometrics were analyzed using IPLab software. Total neurite length was measured; protrusions less than one soma diameter were excluded. Primary and secondary branches were counted. One-way ANOVA analysis with Dunnetts' post hoc tests were used for statistical significance (GraphPad Software, San Diego, CA).

**Western Blot Assay.** Cellular content in the culture was harvested using T-Per protein extraction reagent (Pierce Chemical, Rockford, IL) supplemented with protease inhibitor cocktail, followed by brief sonication and centrifugation. Protein samples from cell lysates were resolved on 4-20% Tris-HCL gels (30 μg/lane) and transferred to polyvinylidene difluoride membrane. Monoclonal antibodies to p-ERK1/2 (1:1000, Cell Signaling Technology), pan-ERK1/2 (1:1000, Cell Signaling Technology) and SNAP-25 (1:5000, Covance, Princeton, NJ) were used to probe for the cognate protein employing a standard western immunoblot protocol. Immunoreactivity was visualized with chemiluminescence and the results were analyzed using Quantity One (BioRad, Hercules, CA).

## RESULTS

BoNTs have demonstrated the ability to inactivate SNARE proteins in multiple neuronal cell types *in vitro*. Under most circumstances, the intact toxin does not readily cross the blood-brain barrier into the CNS due to its large size. Spinal MNs, whose axons innervate the

neuromuscular junction and lack the protection of such a barrier at their nerve terminals, are selectively targeted at extremely low doses of toxin, and therefore, are also highly desirable for toxin studies. Unfortunately, the technical difficulties inherent in harvesting, isolating and culturing primary spinal motor neurons often impede research utilizing primary cells. In this context, the HBG3 cells used in this study proved advantage since the *in vitro* differentiation process induced by RA and Shh recapitulates MN development *in vivo,* and the HBG3-MNs can be readily identified by their spontaneous GFP expression (Wichterle et al., 2002) (Fig. 3.1).

**BoNT/A Promotes Neurite Outgrowth in HBG3-Derived MNs**

To determine the appropriateness of the HBG3 MNs for use in the current study, the effects of BoNT/A on HBG3-MN neurite outgrowth were first investigated. Both neurite length and branching were measured at 6 and 24 hours following exposure to BoNT/A doses ranging from 0.001 nM to 1 nM (Table 3.1). HBG3-MNs displayed significant increases in neurite length and branch numbers in response to BoNT/A treatment, as compared to time-matched, vehicle-treated cells. The examples shown in figure 3.2 illustrate the differences exhibited by vehicle treated MNs and those treated with 1nM BoNT/A for 6 and 24 hours. These responses were qualitatively similar to previous findings in primary embryonic spinal MNs first reported by Coffield and Yan (Coffield and Yan, 2009). Across the dose range, early increases in total neurite length ranged from 24.2-32.0% at 6 hours, while greater increases were observed at 24 hours and ranged from 43.0-57.1%. Increases in primary neurite numbers ranged from 20.7- 22.5% at 6 hours, and 8.2- 47.4% at 24 hours. The most significant effects of BoNT treatment were observed on the number of secondary neurite branches at both time points. Increases at 6 hours ranged from 103.4- 134.1%, whereas those at 24 hours ranged from 73.4- 144.3%. Figure 3.3 illustrates the comparison of the dose-response with vehicle treatment at both time points.

**BoNT/A Induces Phosphorylation of ERK1/2**

The signaling pathways that govern neuritogenesis may vary with cell type, location, culture conditions and numerous other factors. In neurons, the Ras-Raf-MEK1/2-ERK1/2 pathway is essential for cell survival, differentiation, as well as neurite outgrowth (Perron and Bixby, 1999; Frebel and Wiese, 2006; Soundararajan et al., 2010). To determine the involvement, if any, of ERK1/2 signaling in BoNT/A-induced neurite outgrowth, the effect of BoNT/A on ERK1/2 phosphorylation was examined in the cultures of HBG3-MNs treated with 1 nM BoNT/A and compared with time-matched, vehicle treated cells. Cells in the culture were harvested at time points ranging from 30 minutes to 24 hours following the addition of BoNT/A or vehicle to the culture media. Immunoblot detection of pan-ERK1/2 and phosphorylated ERK1/2 was performed following standard western blotting techniques. As shown in figure 3.4, BoNT/A treatment resulted in a transient increase in ERK1/2 phosphorylation that was evident by 30 minutes after treatment initiation, and returned to pretreatment levels by 1 hour. Since phosphorylation of ERK1/2 is associated with its activation, these findings indicate that BoNT/A initiates the activation of the ERK1/2 pathway in the culture of HBG3-MNs within 30 minutes of treatment.

**Inhibition of ERK1/2 Activation Blocks BoNT/A-Induced Neurite Outgrowth**

The role of ERK1/2 signaling in BoNT/A-induced neurite outgrowth was further investigated using the MEK1/2 inhibitor U0126 which prevents activation of ERK1/2. Pre-incubation of HBG3-MN cultures with 10 µM U0126 significantly attenuated the stimulatory effect of 1 nM BoNT/A on neurite outgrowth. As illustrated in figure 3.5, the most significant changes in neurite outgrowth were observed at 24 hours. At this time point, 10 µM U0126 blocked BoNT/A-induced increase in both primary and secondary neurite formation, as well as in total neurite length. The blockade of the toxin effect was essentially complete since all three of the

outgrowth parameters no longer differed from time-matched controls. Partial blockade was evident at 5 µM U0126, in secondary branch numbers at both the 6 and 24 hour time points. These data support a major role for the ERK1/2 pathway in BoNT/A-induced promotion of primary and particularly secondary neurites in cultured HBG3-MNs. Further, toxin-induced secondary neurite formation appears to be the more sensitive feature to inhibition of ERK 1/2 since partial blockade was observed at the lower inhibitor dose and at the earlier time point of 6 hours. Inhibition of ERK1/2 at the higher dose of 10 µM U0126 did have a small but significant effect on total neurite length in the vehicle treated cells but this effect was not present at the lower inhibitor dose. This suggests that ERK1/2 signaling does play a role in basal neurite elongation in HBG3-MNs.

**Inhibition of AKT or PKCα Does Not Affect BoNT/A-Induced Outgrowth**

Both the AKT and PKC pathways have been reported to play roles in neuritogenesis and neuroregeneration. To explore potential roles for these signaling mediators in BoNT/A-induced neurite outgrowth, pathway specific inhibitors were used. The PI3K-AKT cascade was blocked by using the PI3K inhibitor LY296004; while GÖ6976, a classic PKC inhibitor, was used to investigate the role of PKCα. As illustrated by the graphs in figure 3.6, neither 10 µM LY294002 nor 2.3 nM GÖ6976 significantly altered any of the parameters of BoNT/A-induced outgrowth in HBG3-MNs.

**Inhibition of ERK1/2 Does not Alter BoNT/A's Proteolytic Activity**

It is well known that BoNT/A produces paralysis through its proteolytic inactivation of the intracellular SNARE protein SNAP-25 which can be detected by the appearance of a slightly smaller SNAP-25 immunoreactive band (~24 kDa) on a western blot. Previous work in primary motor neurons has shown that the neuritogenic effect of BoNT/A was independent of this SNARE-mediated mechanism (Coffield and Yan, 2009). In the current study, the independence of these

two phenomena was further confirmed. Cell lysates from cultures of HBG3-MNs treated with 1nM BoNT/A in the presence or absence of U0126 were examined by western blot for SNAP-25 cleavage. The presence of a cleaved SNAP-25 band (～24 kDa) was detected in all BoNT/A-treated groups, including those treated with U0126 (Fig. 3.7). The cleavage ratio, which is the density of cleaved SNAP-25 compared to total SNAP25, was approximately 68% and did not differ across the treatment groups. Further, SNAP-25 cleavage was not detected in groups treated with U0126 alone.

**DISCUSSION**

BoNTs are well known for their ability to induce paralysis at the neuromuscular junction with extreme potency (Simpson, 2004; Kiris et al., 2011). It is long established that this paralysis resulted from a blockade of neurotransmitter release from the motor nerve terminals that innervate muscle endplate regions. Over twenty years ago several groups simultaneously determined that the intracellular mechanism leading to the blockade of transmitter release was the enzymatic cleavage by BoNTs of SNARE proteins necessary for synaptic vesicle fusion and transmitter release (Schiavo et al., 1992; Blasi et al., 1993; Binz et al., 1994). A less well studied, secondary outcome associated with BoNT poisoning was the ‘sprouting’ of neurites from affected terminals. These neurites were reported to transiently innervate the quiescent endplate region and possibly initiate recovery. The cellular mechanism responsible for this toxin-induced sprouting received little attention. Subsequently, Coffield and Yan (2009) reported that BoNT/A stimulated neurite outgrowth in primary cultures of spinal motor neurons, eliminating muscle as a potential source of an initiating factor. While the mechanism of this *in vitro* sprouting phenomenon was not clarified, the authors determined that the action was independent of the known effect of BoNT/A on the

SNARE protein SNAP-25. In the current paper, we report that BoNT/A promotes neurite outgrowth in cultured motor neurons by activating the ERK1/2 pathway. To investigate this phenomenon, we studied HBG3-MNs, motor neurons that were derived from mouse stem cells.

HBG3-MNs can be successfully differentiated from embryonic stem cells at a high ratio (Wichterle et al., 2002). Similar to primary mouse MNs, the differentiated HBG3 neurons have been shown to express choline acetyltransferase, SNAP-25, synaptic vesicle glycoprotein 2A and the transcription factor Hb9, a motor neuronal marker (Wichterle et al., 2002; Kiris et al., 2011). HBG3-MNs have also been reported to populate into the spinal cord and innervate target muscles following introduction. These characteristics, as well as their demonstrated sensitivity to BoNT intoxication originally reported by Kiris et al (2011) and confirmed here, suggest that HBG3- MNs may be useful candidates for in vitro BoNT studies. In fact, when used in the current neurite outgrowth study, HBG3-MNs showed qualitatively similar responses to BoNT as did primary motor neurons, e.g. increased total neurite length and numbers of primary and secondary branches. One noticeable difference was that the HBG3-MNs grew faster than primary cells and therefore there was a corresponding shift in the outgrowth response to BoNT toward earlier time points.

Neurite outgrowth is an important physiological process that establishes intercellular connections during both neural development and post-injury recovery. In addition to the critical roles of extracellular factors, researchers have noted that some intrinsic factors, such as Bcl-2, could also control neurite outgrowth (Holm and Isacson, 1999). In fact, a number of signaling pathways have been reported to regulate neurite outgrowth; although, the specific neuritogenic mechanisms at play in motor neuron development and/or regeneration are largely unknown (Ozdinler and Macklis, 2006; Aglah et al., 2008; Soundararajan et al., 2010; Newbern et al., 2011). ERK1/2 is an important signaling kinase that belongs to the MAPK family (Roskoski, 2012). It is

a threonine/tyrosine kinase, both of which need to be phosphorylated in order to convert the kinase to its active form. Typically, activation of the ERK1/2 pathway is initiated following the activation of a tyrosine kinase receptor on the cell membrane, leading to activation of the Ras-Raf-MEK1/2-ERK1/2 signaling cascade, and subsequent modification of relevant target molecules. MEK1/2 are the kinases required for ERK1/2 phosphorylation and activation. Activation of this pathway regulates many cellular responses including, but not limited to, adhesion, metabolism, transcription, proliferation, differentiation and migration. In addition to these diverse roles, specific patterns of ERK1/2 activation can sometimes lead to opposing cellular responses (Ebisuya et al., 2005). In neurons, the ERK1/2 pathway is essential for cell survival and differentiation, as well as neurite outgrowth (Perron and Bixby, 1999; Frebel and Wiese, 2006; Soundararajan et al., 2010).

The ERK1/2 pathway has been postulated to be a point of convergence for different signaling pathways regulating neuritogenesis (Perron and Bixby, 1999). Soluble growth factors (eg. bFGF), as well as adhesions molecules (e.g. laminin or N-cadherin) have all been shown to employ ERK1/2 in downstream signaling to induce neurite growth. In addition, ERK1/2 mediated neurite outgrowth has been demonstrated in neurons of different origin, such as retinal neurons and cortical neurons, as well as stem cell-derived neurons (Perron and Bixby, 1999; Qiu et al., 2004; Harrill et al., 2010). The ERK1/2 pathway potentially mediates fibroblast growth factor induced neurite extension and axon guidance, as well as injured motor nerve regeneration (Aglah et al., 2008; Agthong et al., 2009; Soundararajan et al., 2010).

Results from studies involving ERK1/2 or MEK1/2 manipulation in MNs are limited. A drug used to treat epilepsy and Parkinson’s disease, was reported to promote neurite elongation and regeneration in MNs, but suppressed the phosphorylation of ERK1/2 (Yagi et al., 2015). In the current study, the MEK1/2 inhibitor reduced total neurite length and secondary branch

formation when applied with BoNT/A. Interestingly, primary neurite formation was not altered in the presence of the MEK1/2 inhibitor alone, whereas, the inhibitor did block the stimulatory effect of BoNT/A on primary neurite formation. In addition, BoNT/A evoked ERK1/2 phosphorylation in cultures of HBG3-MNs. Collectively, these data confirm that the ERK1/2 pathway is critical for toxin-induced neurite outgrowth in HBG3-MNs. These results are in general agreement with previous research indicating a crucial role for ERK1/2 in neuritogenesis. Furthermore, the differential effects of the MEK inhibitor on primary and secondary neurite formation, either in the presence or absence of BoNT/A, suggest that alternate pathways regulate primary neurite formation in MNs (Shelly et al., 2010).

In addition to the ERK1/2 pathway, the potential roles of the AKT and PKC pathways in neuronal development and repair were also studied. For example, AKT has been associated with adult MN regeneration, although studies on the exact downstream targets which differentiate this role from other cellular responses are limited (Namikawa et al., 2000; Read and Gorman, 2009). PKC has been shown to have complex actions. It seems to mediate neurite elongation in MNs; conversely, outgrowth inhibitory factors in the CNS, such as the chondroitin sulfate proteoglycans, are also known to act via the PKC pathway (Guzman-Lenis et al., 2009; Usher et al., 2010). To examine the direct effects of these pathways on toxin-induced neuritogenesis, pharmacological inhibitors were employed with BoNT/A and neurite outgrowth assays were performed. Although overexpression of AKT is known to promote MN regeneration, loss of function of AKT by LY294002 at doses not affecting survival did not impact BoNT/A-induced neurite outgrowth, suggesting that the toxin-stimulated neurite outgrowth in HBG3-MNs is independent of the AKT pathway. Similar results were found with GÖ6976. Although preliminary study indicated that PKCα mRNA was increased slightly following BoNT/A exposure (data not shown), GÖ6976 used

at a dose reported to block PKCα had no effect. One thing worth noting, while 10 μM LY294002 has been reported to inhibit AKT phosphorylation in neurons by others, phosphorylation of AKT in the presence of LY294002 was not examined in the current study. Thus, further studies are needed to confirm the findings reported herein (Endo et al., 2006; Guo et al., 2011; Cheng et al., 2013). Similarly, examining the phosphorylation of PKC as a measure of activation would strengthen the current findings (Zhang et al., 2006; Zhou et al., 2006). The activation of PKC involves two steps that contribute equally (Newton, 1995). First, PKC needs to be phosphorylated and then substrate comes in to remove the pseudosubstrate from the binding site. Activated PKC further phosphorylates its substrates to exert a cellular response. Comparing the degree of substrate phosphorylation is another way to measure PKC activity (Koivunen et al., 2004; Zhou et al., 2006). Although the doses for GÖ6976 used in the current study have been tested by the manufacturer and reported by others to block PKC in several cell types, many published research in neurons tested much higher doses (0.1 to 2 μM), suggesting that a dose response experiment covering a wider range in the HBG3-MNs cultures will be necessary to confirm the findings of the current study (Zhang et al., 2006; Wang et al., 2014; Higa-Nakamine et al., 2015; Xu et al., 2015).

To date, studies of the intracellular actions of BoNT/A have centered on the proteolysis of SNAP-25 and the resultant blockade of transmitter release that leads to paralysis. This is the first study to report that an additional major action of BoNT/A, activation of a crucial intracellular signaling pathway, the ERK1/2 pathway, occurs downstream of BoNT/A exposure. While it has been shown that these two intracellular mechanisms of BoNT/A occur independently of one another, this does not mean that their activities are not inter-related. In fact, it is well known that BoNT/A exposure leads to a particular form of secondary branching *in vivo*, *aka*, axonal sprouting, at poisoned NMJs (de Paiva et al., 1999). Moreover, this activity is thought to initiate recovery

from the paralysis caused by BoNT/A. Conversely, this sprouting phenomenon is one potential reason for the diminishing efficacy of BoNT/A's therapeutic effect over time, when used to treat various dystonias, and resulting in repeated injections of BoNT/A, which are not without risk (Harrison et al., 2011). Limited study has been done on potential mechanisms underlying this sprouting response. A few reports indicated that extracellular molecules derived from affected muscle, such as insulin growth factor (IGF), could partially mediate the sprouting observed at BoNT-paralyzed motor endplates (Caroni and Schneider, 1994; Harrison et al., 2011). However, including the results of the current study, it has now been demonstrated that 'sprouting' in motor neurons can occur independently of muscle related factors (Coffield and Yan, 2009). Finally, additional results reported here demonstrate that SNAP-25 cleavage was not altered in the presence of the MEK inhibitor, indicating that inhibition of the ERK1/2 pathway does not interfere with BoNT/A's protease activity; therefore, its established therapeutic properties remain intact. Collectively, these findings support the importance of pursuing future studies to investigate mechanisms for manipulating BoNT/A-induced sprouting in efforts to enhance recovery from paralysis or improve the toxin's therapeutic efficacy. Further, these results encourage additional studies to investigate the use of BoNT/A in neurodevelopment and regeneration.

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Table 3.1. Comparative changes in neurite outgrowth following BoNT/A treatment.

| Percent Change in Neurite Outgrowth | | | | | |
|---|---|---|---|---|---|
| **Parameter** | | **BoNT/A (nM)** | | | |
| | | 0.001 | 0.01 | 0.1 | 1.0 |
| Neurite Length | 6h | 32.0%** | 29.0%** | 29.2%** | 24.2%* |
| | 24h | 44.0%*** | 43.0%*** | 57.1%*** | 56.8%*** |
| Primary Branches | 6h | 21.9%** | 20.7%** | 21.9%** | 22.5%** |
| | 24h | 8.2% | 35.2%** | 47.4%*** | 41.3%*** |
| Secondary Branches | 6h | 117.6%*** | 103.4%*** | 133%*** | 134.1%*** |
| | 24h | 73.4%*** | 113.3%*** | 125.8%*** | 144.3%*** |

HBG3-MNs were treated with BoNT/A (0.001 nM to 1 nM) for 6 and 24 hours. Comparisons were made with time-matched controls (0 nM). Numbers indicate percent increase above control values. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, compared with 0 nM BoNT/A.

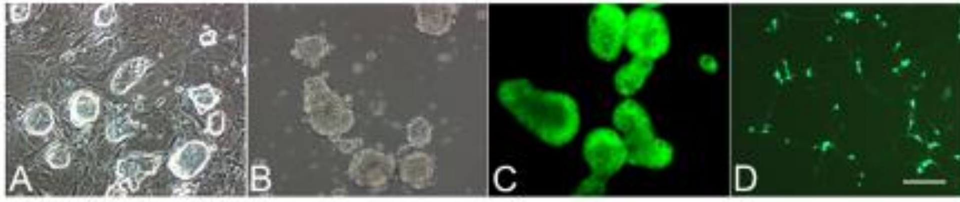


Figure 3.1: Proliferation and differentiation of HBG3 cells. (A) Colonies of HBG3 stem cells formed on MEF layer after 3 days in culture. (B) EBs in aggregate culture at D2. (C) EBs in aggregate culture at D5. (D) GFP+ MNs plated on matrigel coated plates 1 day after EB dissociation. Scale bar, 100 µm.

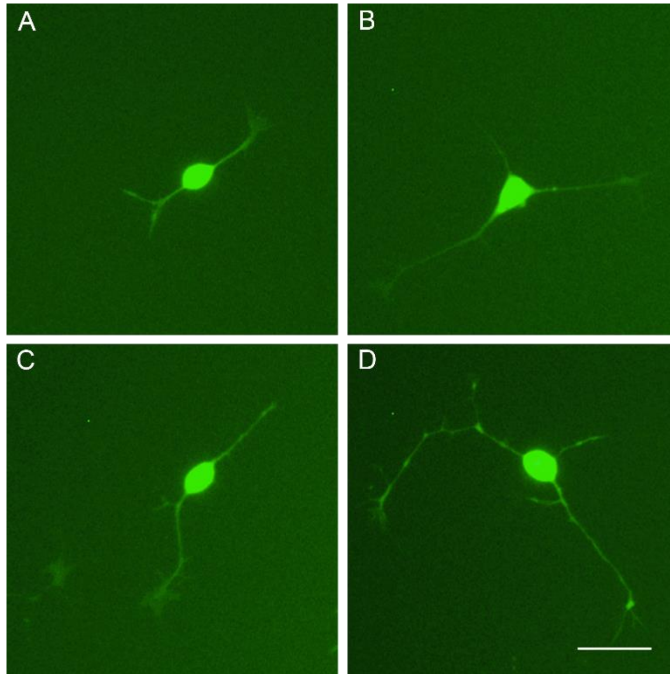


Figure 3.2: Morphological comparison of HBG3 MNs after exposure to BoNT/A. Fluorescent images of GFP+ HBG3-MNs treated with BoNT (1 nM) or without at 6 and 24 hours. (A) Control MNs, 6 hours. (B) BoNT/A-treated, 6 hours. (C) Control, 24 hours. (D) BoNT/A-treated, 24 hours. Scale bar, 50 µm.

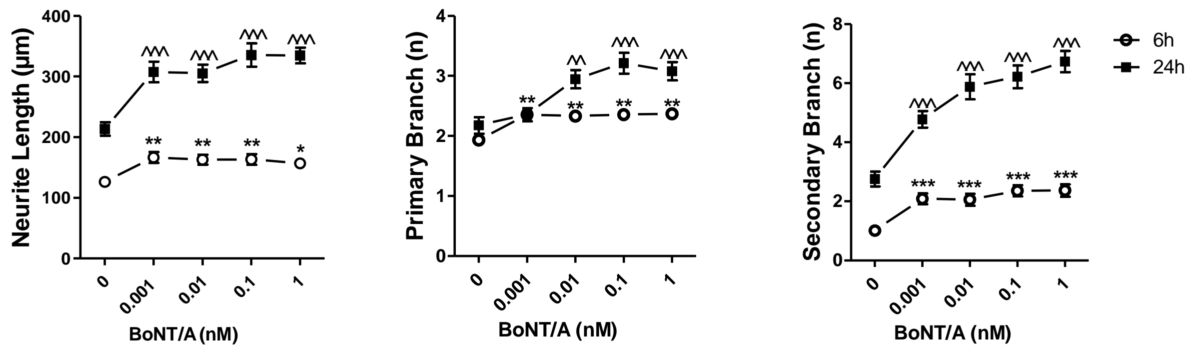


Figure 3.3: Dose-dependent effects of BoNT/A (0.001 nM to 1 nM). Comparison of total length, primary and secondary neurite numbers at 6 hours (filled circle) and 24 hours (filled square). (mean ± S.E.M., n=90). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, compared with 0 nM BoNT/A at 6 hours. ^^$P < 0.01$ and ^^^$P < 0.001$, compared with 0 nM BoNT/A at 24 hours.

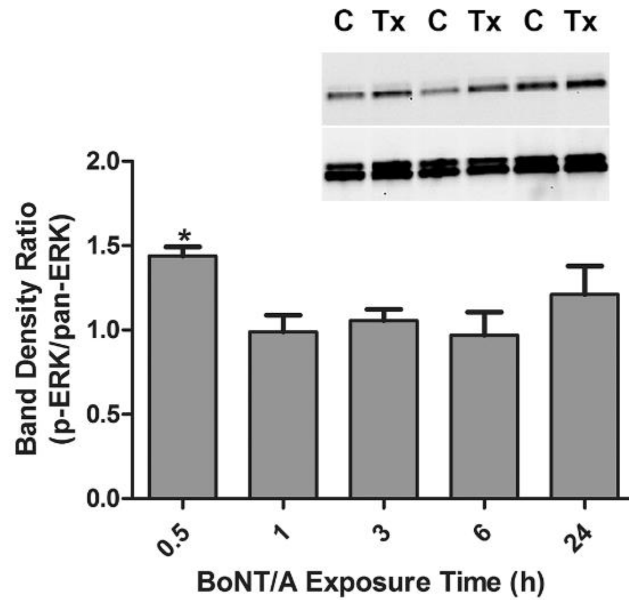


Figure 3.4: ERK1/2 Phosphorylation is enhanced upon BoNT/A Treatment in cultures of HBG3-MNs. (A) Immunoblot of p-ERK1/2 and pan-ERK1/2 from HBG3-MNs after BoNT/A exposure from 30 minutes to 24 hours. (B) Comparison of relative band density (p-ERK/pan-ERK) with time-matched control (mean ± S.E.M., n=3). *$P < 0.05$, compared with time-matched controls.

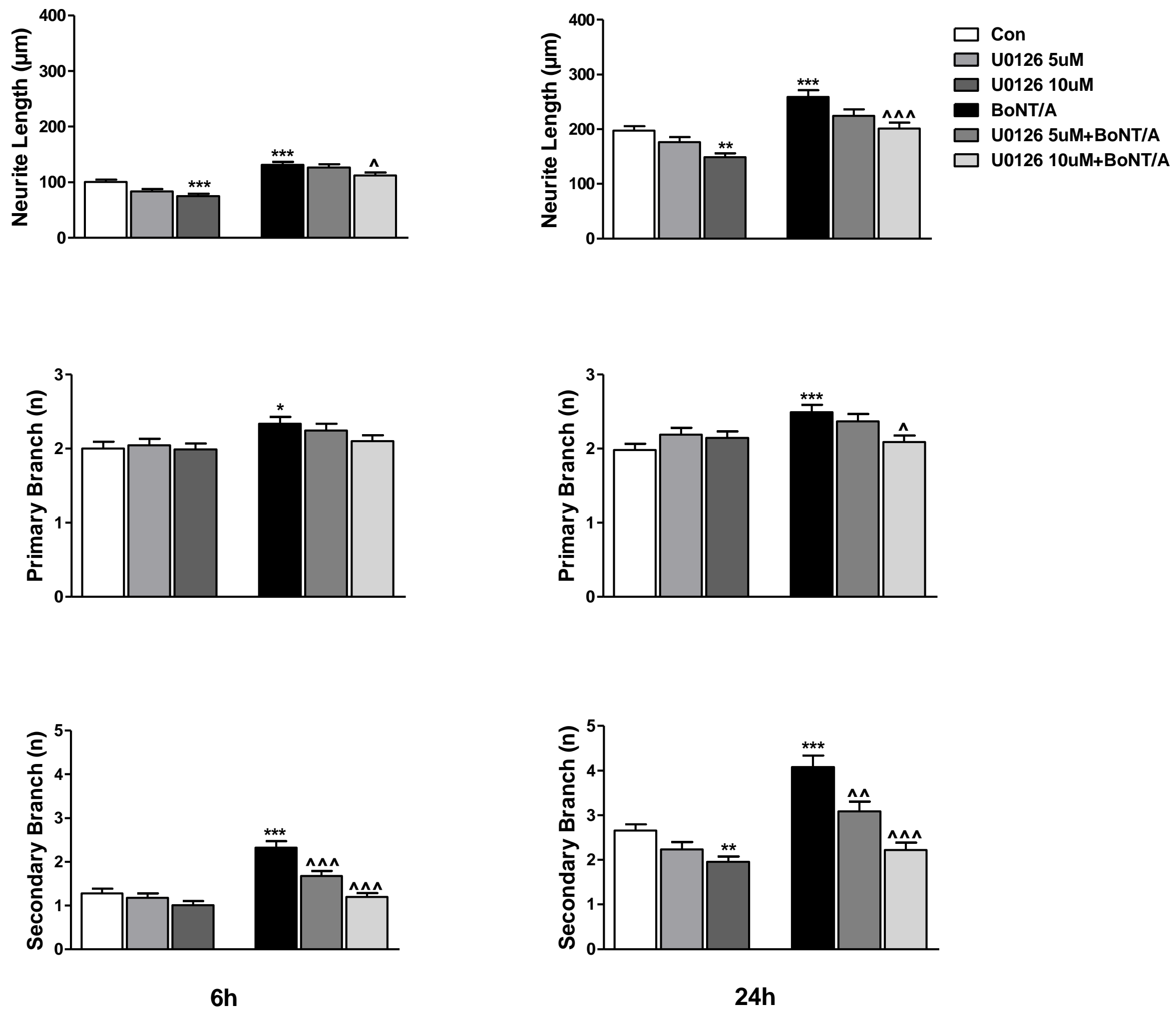


Figure 3.5: Effects of U0126 on BoNT/A-induced neurite outgrowth. Comparison of total length, primary and secondary neurite numbers (mean± S.E.M., n=90). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$, compared with controls. ^ $P<0.05$, ^^ $P<0.01$ and ^^^ $P<0.001$, compared with 1 nM BoNT/A.

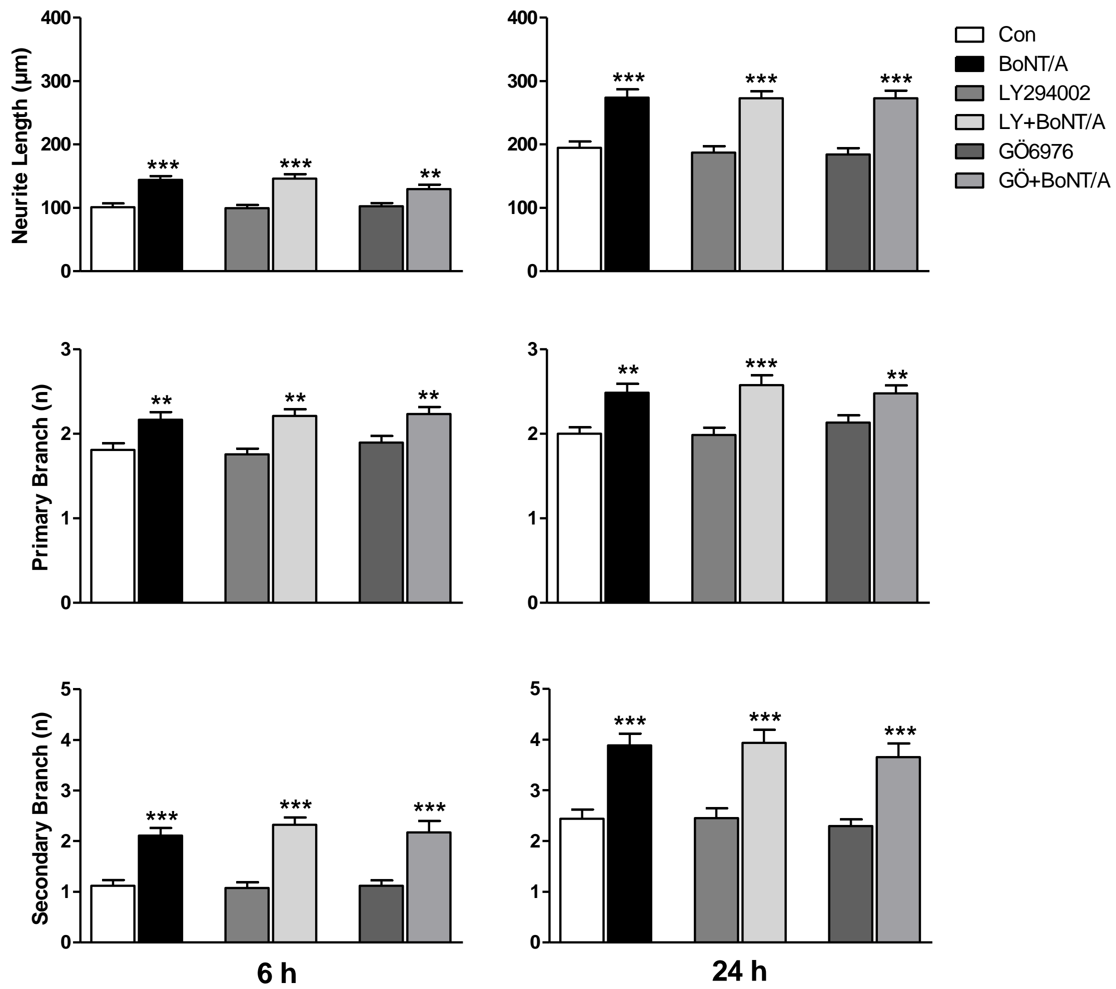


Figure 3.6: Effects of LY294002 and GÖ6976 on BoNT/A-induced neurite outgrowth. Comparison of total length, primary and secondary neurite numbers (mean± S.E.M., n=90). ** *P*<0.01 and *** *P*<0.001, compared with controls.

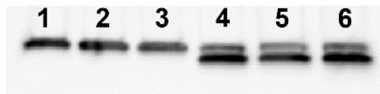


Figure 3.7: Immunoblot of SNAP-25 protein from cells in the HBG3-MN culture isolated at 24-hours post exposure. Band 1, control or no exposure; band 2, U0126 5 nM; band 3, U0126 10 nM; band 4, BoNT/A 1 nM; band 5, U0126 5 nM+ BoNT/A 1 nM; band 6, U0126 10 nM+ BoNT/A 1 nM. Presence of two bands in band 4, 5 and 6 indicates SNAP-25 cleavage, with the lower band represents cleaved SNAP-25.

# CHAPTER 4

# BRAIN DERIVED NEUROTROPHIC FACTOR PLAYS A CRITICAL ROLE IN BOTULINUM NEUROTOXIN A INDUCED NEURITOGENESIS[1]

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## ABSTRACT

Botulinum neurotoxin (BoNT) is known to cause blockade of neurotransmitter release at cholinergic synapses with extremely high potency. Although potentially fatal, the debilitating disease botulism is conceivably recoverable with extensive supportive care. This recovery is thought to be initiated by axon terminal sprouting, a form of neurite outgrowth, at the paralyzed neuromuscular junctions. Interestingly, ongoing studies in our laboratory have demonstrated that BoNT serotype A (BoNT/A) induces neurite outgrowth in mouse embryonic motor neurons (MNs) as well as HBG3 stem cell derived MNs (HBG3-MNs). The current study aimed to examine the involvement of brain derived neurotrophic factor (BDNF) in BoNT/A induced neurite outgrowth. Preliminary study in cultured embryonic primary MNs using RT-PCR microarrays analysis revealed that *bdnf* is elevated in response to BoNT/A exposure. Further, quantitative PCR confirmed the upregulation of *bdnf* in cultures of HBG3-MNs following BoNT/A exposure. When exogenous BDNF was bath applied to the HBG3-MN culture, it promoted secondary neurite formation and increased total neurite length, in a manner similar to the response following BoNT/A exposure. To examine the direct interaction between endogenous BDNF and BoNT/A-induced outgrowth, HBG3-MNs cultures were pre-treated with a BDNF scavenger TrkB-Fc prior to the exposure of BoNT/A. Interestingly, TrkB-Fc showed nearly complete blockade of the stimulatory effect of BoNT/A on primary and secondary neurite formation, as well as total neurite length. Additionally, inhibition of the extracellular signal-regulated kinases 1/2 (ERK1/2) pathway blocked BoNT/A induced *bdnf* upregulation. In summary, these data suggest that BoNT/A induces neurite outgrowth through upregulation of neurotrophic factors like BDNF, and this upregulation may be mediated by the ERK1/2 pathway.

**Key Words:** BoNT/A, neurite outgrowth, BDNF, HBG3-MNs, ERK1/2

## INTRODUCTION

Botulinum neurotoxin (BoNT) is the most potent biotoxin present in nature, and is classified by the CDC as one of the six "Tier 1" agents (Centers for Disease et al., 2012). There are seven serotypes of the deadly neurotoxin (A to G) with A, B, E and F being the primary agents responsible for human botulism, a potentially fatal disease characterized by flaccid muscle paralysis (Sobel, 2005). BoNT causes blockade of cholinergic neurotransmitter release at neuromuscular junctions with extremely high potency, and has an estimated lethal dose for a 70 kg human of around 0.09 - 0.15 μg when given intravenously. Although potentially fatal, the debilitating disease botulism is conceivably recoverable with extensive supportive care. Despite its extreme toxicity, BoNT serotype A (BoNT/A) has been used clinically to treat a broad range of conditions from uncontrolled muscle spasms (e.g. blepharospasm, dystonias) to undesirable wrinkles. Although there is no cure for botulism, the potential for recovery is thought to be contingent upon the initiation of sprouting from axon terminals at the paralyzed neuromuscular junctions (de Paiva et al., 1999). Of note, this same sprouting phenomenon may also account for the need for repeated injections of BoNT/A used therapeutically.

The cause of sprouting under these conditions has not been extensively investigated. One study reported the involvement of insulin growth factors derived from the inactive muscle fibers following toxin treatment (Caroni and Schneider, 1994). A different study reported enhanced neurite outgrowth in purified embryonic MN cultures, suggesting that the sprouting phenomenon induced by BoNT/A treatment was the result of some unknown intra-neuronal mechanisms (Coffield and Yan, 2009). The intracellular molecular mechanism that leads to BoNT/A-induced paralysis is known to be the proteolytic cleavage of the SNARE protein SNAP-25 by the enzymatic domain of the toxin. However, other mechanism(s) must be responsible for BoNT/A induced

neurite outgrowth since neurite outgrowth still occurs in the presence of the toxin's binding domain alone which cannot produce SNAP-25 cleavage in the absence of the enzymatic domain. Few BoNT/A studies have examined the potential involvement of signaling transduction pathways in toxin action, not to mention the pathways directly leading to sprouting. Interestingly, since BoNT/A induced neurite outgrowth was blocked by the ERK1/2 pathway (chapter 3), and ERK1/2 signaling served as an downstream effector of the brain derived neurotrophic factor (BDNF) in MNs (chapter 2), it was of interest to determine if there are any direct connections between BoNT/A and BDNF. As a member of the neurotrophin family, BDNF is crucial for the development and function of nervous systems. Research has shown a direct effects of BDNF on motor neurons (MNs), and BDNF administration is associated with reduced MN degeneration from injury, and improved axonal regeneration (Kishino et al., 1997; Naeem et al., 2002). Enhancing BDNF levels is one potential way to promote the recovery of MN injury or disease (Ying et al., 2005; Yamauchi et al., 2006; Boyce et al., 2007). Thus, it is hypothesized that BDNF might be involved in BoNT/A-induced neurite outgrowth and merits further examination.

In the present study, PCR array technology was initially used to screen for potential gene alterations in a number of signaling pathways in cultured mouse primary embryonic MNs treated with BoNT/A. The findings revealed that *bdnf* was upregulated in the toxin-treated primary MN cultures. These initial findings were then confirmed using real-time PCR in cultures of HBG3 stem cell-derived motor neurons (HBG3-MNs), indicating that *bdnf* was up-regulated following exposure to BoNT/A. Further, the increased *bdnf* expression in the culture of HBG3-MNs coincided with BoNT/A induced neurite outgrowth. When BDNF protein was added exogenously to HBG3-MN cultures it induced secondary neurite formation, confirming both BDNF's well established role in neuritogenesis as well as the sensitivity of the HBG3-MNs to BDNF (Martin et

al., 2012). To further test the functional relationship between BoNT/A and BDNF, neurite outgrowth assays were conducted in the presence of both BoNT/A and the BDNF scavenger TrkB-Fc. TrkB (tropomyosin kinase receptor B) is the membrane receptor for mature BDNF on the neuronal membrane. The Fc fragment of TrkB when applied exogenously can be used as a soluble ligand for secreted BDNF, thereby disrupting BDNF signaling through the BDNF-TrkB receptor (Cheng et al., 2011). The results showed that TrkB-Fc not only blocked the stimulatory effects both BNDF and BoNT/A on neurite length and secondary branch formation, it also blocked the specific stimulatory action of BoNT/A on primary neurite formation. Moreover, BoNT/A induced BDNF up-regulation was blocked by inhibition of the extracellular signal-regulated kinases 1/2 (ERK1/2) pathway.

In summary, the findings presented in the current study support the hypothesis that BDNF mediates BoNT/A induced neurite outgrowth in MNs, particularly the toxin-induced increase in secondary branch formation. The up-regulation of *bdnf* is possibly regulated by the ERK1/2 pathway. However, since BoNT/A induces both primary and secondary neurite formation, additional studies are necessary to identify additional mediators involved in primary branch formation.

## MATERIALS AND METHODS

### RT-PCR Microarray Analysis

All procedures involving animals were approved by the Institutional Animal Care and Use Committee. Animal surgery and MN isolation were carried out as previously described (Coffield and Yan, 2009). MNs were seeded to plates coated with poly-D-lysine plus laminin (BD Biosciences, San Jose, CA) in L-15 media (Gibco, Grand Island, NY) supplemented with 10%

glucose, 2 mM L-glutamine (Gibco), 5% horse serum, 7.5% sodium bicarbonate, 1% penicillin-streptomycin (Gibco), 5 µg/ml insulin, 100 µg/ml putrescine, 100 µg/ml conalbumin 3 µM sodium selenite, and 2 µM progesterone. Following 4 hours of attachment, mouse embryonic MNs were treated with 1 nM BoNT/A (Metabiologics, Madison, WI) for 12 hours. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA) with On-Column DNase (Qiagen) digestion. cDNA was generated using the $RT^2$ First Strand synthesis kit (Qiagen) and a PTC-200 Peltier Thermal Cycler (Biorad, Hercules, CA). The cDNA synthesis reaction (amount equals to 400 ng total RNA), SYBR green mastermix (Qiagen) and RNase-free water were loaded into three 384-well microarray pathway plates including the Signal Transduction PathwayFinder, Focal Adhesions, and Neurotrophins and Receptors (Qiagen). $RT^2$-PCR was performed using the ABI 7900HT cycler following manufacturer's instructions. Three biological replicates were used for each study. All data were analyzed using Qiagen $RT^2$ Profiler PCR Array Data Analysis software version 3.5.

**HBG3 Stem Cell Differentiation and MN Culture**

HBG3 cells have an *eGfp* gene introduced into the promoter region of the *Hb9* gene, which is a known post-mitotic marker of MNs (Wichterle et al., 2002). Once HBG3 cells are differentiated into MNs, the cultured cells display green fluorescence spontaneously and can be detected by standard fluorescence microscopy without staining. MN differentiation was performed as described previously. In brief, at day 0 (D0), HBG3 mouse embryonic stem cells (mESC) were cultured in ADFNK medium, which is a 1:1 mixture of Advanced DMEM/F12 medium (Gibco) with Neurobasal medium (Gibco), supplemented with 10% Knockout serum replacement (Gibco), 2 mM L-glutamine with penicillin/streptomycin and 0.1 mM β-mercaptoethanol (Gibco). After 2 days in aggregate culture, medium was supplemented with 1 µM retinoic acid (RA, Sigma-Aldrich,

St. Louis, MO) and 100 ng/ml sonic hedgehog (Shh, R&D Systems, Minneapolis, MN). At D5, the medium was further supplemented with 10 ng/ml glial-derived neurotrophic factor (GDNF, Biosource International, Camarillo, CA Biosource) and 2 ng/ml ciliary neurotrophic factor (CNTF, Biosource International). At D6, embryoid bodies were dissociated with 0.05% trypsin-EDTA (Gibco) for 4 minutes at 37˚C. Cells were then seeded in 24-well plates coated with matrigel (BD Biosciences, San Jose, CA).

**Real-time PCR**

MN-enriched culture systems were treated with 1 nM pure BoNT/A alone, or 10 μM U0126 (Cell Signaling Technology, Danvers, MA), or both with U0126 added 30 minutes before toxin. Real-time PCR was performed using a Stratagene Mx3005P qPCR machine. Expression of *bdnf* were quantified and normalized by *gapdh*.

**Neurite Outgrowth Assay**

Cells were cultured in ADFNK medium supplemented with 10 ng/ml GDNF, 2 ng/ml CNTF, 2% B27 (Gibco) and 1% N2 (Gibco), and plated at a density of 33,000 cells per well. After 30 minutes of attachment, cells were exposed to media containing either 50 ng/ml BDNF (Biosource, Camarillo, CA), 1 nM BoNT/A, 2 µg/ml TrkB-Fc, 1 nM BoNT/A plus 2 µg/ml TrkB-Fc (R&D Systems). For the latter combination, TrkB-Fc was added 30 minutes after BoNT/A to allow toxin internalization. Cell images were captured 6 and 24 hours after the final treatment.

GFP-positive neurons were visualized using an Olympus (Tokyo, Japan) inverted microscope. Cells meeting the following criteria were captured in a grid pattern with MagnaFIRE SP software using a grid pattern: 1) cells showing strong GFP intensity; 2) length of the longest neurite not less than two times the soma diameter; 3) somata displaying a pyramidal like morphology. Ninety neurons from three replicate experiments were analyzed per treatment group

using IPLab software. Total neurite length was measured; protrusions less than one soma diameter were excluded. Primary and secondary branches were counted. One-way ANOVA analysis with Dunnetts' post hoc multiple comparison was used to test for statistical significance (GraphPad Software, San Diego, CA).

## RESULTS

### BoNT/A Enhances *bdnf* Gene Expression

Besides the known neurotrophins, neuroprotective agents that directly induce neuritogenesis are scarce and interventions for neurodegenerative diseases remain a challenging area (De Felice and Ferreira, 2006). This laboratory has previously reported that BoNT/A promotes neurite length and primary/secondary branch formations in mouse primary MNs, through undetermined mechanisms (Coffield and Yan, 2009). In the current study, PCR microarray analyses were used in to initially screen for possible gene alterations upon BoNT/A treatment. Mouse embryonic primary MNs were cultured and exposed to BoNT/A for 12 hours. After RNA extraction and cDNA preparation, samples were loaded onto 3 categorized microarray plates targeting 252 genes related to focal adhesion, neurotrophins and receptors, and signal transduction pathways. Results showed that the expression of a few genes were significantly altered by BoNT/A treatment (Table 4.1, $P<0.1$). Among the altered genes, *bdnf* was chosen for further investigation because of its established functions in neuronal and neurite development.

To confirm the upregulation of *bdnf* by the BoNT/A, cultures of HBG3-derived MNs were treated with 1 nM of BoNT/A. Concurrent studies in our lab (chapters 2 and 3 of this dissertation) as well as the study by Kiris et al. indicate that these stem cell-derived MNs are a useful model for BoNT/A study (Kiris et al., 2011). Samples were collected for real-time PCR analysis of total

RNA from 30 minutes to 24 hours following toxin treatment. As illustrated in Figure 4.1, *bdnf* gene expression was significantly enhanced between 6 to 24 hours, with a fold change of 1.3.

**Exogenous BDNF Induces Neuritogenesis of HBG3-MNs**

BDNF is known to promote the survival of MNs and induce dendritogenesis (Huang and Reichardt, 2001; Martin et al., 2012). Interestingly, it is believed that different subtypes of MNs respond differently to neurotrophins. To test the responsiveness of HBG3-MNs to BDNF, neurite outgrowth was analyzed in the presence of 50 ng/ml BDNF added to culture media and compared to vehicle-treated, time-matched controls. Two time points were selected for observation. The results revealed that BDNF application increased total neurite length and secondary branch formation at both 6 and 24 hours, while the numbers of primary branches were not altered (Fig. 4.2). These results indicate that HBG3-MNs respond vigorously to extracellular BDNF. Interestingly, BDNF application had no effect on primary neurites, in contrast to BoNT/A which enhances the formation of both primary and secondary neurites.

**U0126 Blocks BoNT/A-Induced *bdnf* Up-Regulation**

Activation of the ERK1/2 signaling pathway is reported to induce *bdnf* expression (Takayama and Ueda, 2005; Matsuoka and Yang, 2012; Okuyama et al., 2012). Results from concurrent studies presented in chapters 2 and 3 of this dissertation demonstrate that in the cultures of HBG3-MNs the ERK1/2 pathway is activated by both BoNT/A and BDNF. In addition, these studies found that ER1/2 signaling plays a critical role in neurite outgrowth induced by BoNT/A. In the current study, when the inhibitor U0126, which blocks ERK1/2 phosphorylation and activation by inhibiting the mitogen-activated protein kinase kinase 1/2 (MEK1/2), was applied to the culture of HBG3-MNs in the presence of BoNT/A, the toxin-induced up-regulation in *bdnf* expression was eliminated. This finding confirms the critical role of ERK1/2 activation in BoNT/A

induced neurite outgrowth and suggests that BDNF plays a pivotal role in that outgrowth pathway (Fig. 4.3).

**TrkB-Fc Reverses BoNT/A-Induced Neuritogenesis**

BoNT/A and exogenous BDNF both induce neuritogenesis in HBG3-MNs. To test whether endogenous BDNF is a mediator for BoNT/A induced neuritogenesis, TrkB-Fc (2 μg/ml) was bath applied to cultured HBG3-MNs in the presence of 1 nM BoNT/A (Fig. 4.4). In preliminary experiments, TrkB-Fc at 2 μg/ml blocked the neuritogenic effect induced by exogenous BDNF and was selected for use in this study. As reported in chapters 3, BoNT/A alone increases the formation of primary and secondary neurites, as well as total neurite length. However, when TrkB-Fc was applied 30 minutes after the application of BoNT/A, the BDNF scavenger blocked all aspects of the toxin-induced neuritogenesis nearly completely. TrkB-Fc by itself had no significant effect on neurite outgrowth in the HBG3-MNs. Interestingly, TrkB-Fc blocked not only secondary branch formation induced by BoNT/A, but also primary branch formation.

## DISCUSSION

Independent of the cellular mechanism that causes botulism, BoNT/A has been found to promote neurite outgrowth of MNs (de Paiva et al., 1999; Coffield and Yan, 2009). The cellular mechanism(s) underlying this phenomenon remain unknown. A few studies indicate that muscle fiber-derived extracellular molecules, like insulin growth factor (IGF), might partly mediate the sprouting process observed in BoNT-paralyzed muscles (Caroni and Schneider, 1994; Harrison et al., 2011). These findings are based on the assumption that sprouting relies on the activity of neighboring muscles. However, one study showed that BoNT/A induced neuritogenesis in the absence of muscle fibers, implying that some neuronal factors play critical roles in this process

(Coffield and Yan, 2009). No study has explored muscle-independent signaling profile upon BoNT/A treatment so far.

Only limited study has been done to examine the effect of BoNT/A exposure on gene expression. One microarray study done in epithelial and neuronal cell lines demonstrated that BoNT/A induces diverse changes in the whole genome expression (Thirunavukkarasusx et al., 2011). In the current study, results in table 4.1 demonstrate that the changes in gene expression after exposure are minor in primary MNs at 12 hours, similar to one recent study done in induced pluripotent stem cell-derived human neurons at 2 days (Scherf et al., 2014). Moreover, Scherf suggested that the altered genes are involved in neuritogenesis and calcium channel signaling, and these gene alterations induced by BoNT/A were similar to to those by a catalytically inactive form of BoNT/A. Among the significantly altered genes from our primary MN assay, *bdnf* was recognized to have direct effects on motor neuron differentiation. As reported in chapter 3, BoNT/A induced neuritogenesis in HBG3-MNs, in a manner similar to primary MNs. Here it was confirmed that BoNT/A also enhanced the expression of *bdnf* in the culture of HBG3-MNs, allowing us to further explore more detailed mechanisms with this model of MNs.

Some other significantly altered genes, as illustrated in table 4.1, have also been reported to promote neurite development. For example, cytokines such as interleukin 2 (Il2) was reported to promote neurite elongation and branching in cultured hippocampal neurons with unknown mechanisms (Sarder et al., 1996). Leptin, a hormone that regulates energy homeostasis, facilitated long term potentiation in hippocampal neurons, and increased the size of growth cones in axons of cortical neurons (Valerio et al., 2006; O'Malley et al., 2007; Quarta et al., 2014). PKCα has been reported to play roles in neurite outgrowth in hypothalamic neurons, while from our data in chapter

2 PKCα might mediate cellular response other than neurite outgrowth in motor neurons (Choe et al., 2002). Additional studies are warranted to explore the effect of these altered genes.

BDNF, a member of the neurotrophic factor (NTF) family, is crucial for the development and function of nervous systems (Kernie et al., 2000). It supports neural tube development and neuronal precursor cell differentiation during embryogenesis. Later in the mature nervous system, BDNF regulates neuronal branching, remodeling and plasticity. BDNF gene organization is conserved among species but complex, with 10 alternative exons in human, one exon coding for pro-BDNF protein, and multiple promoters, in order to achieve an activity-dependent regulation of transcription (Cohen-Cory et al., 2010). Translation of *bdnf* mRNA is also tightly regulated: the mRNA can remain untranslated in dendrites until stimulated. BDNF is initially synthesized as a large precursor protein known as pro-BDNF, then undergoes proteolytic cleavage to form mature BDNF, although both exist intracellularly and can be secreted. Secretion of BDNF is mediated by two distinct ways: a constitutive pathway that occurs in all types of cells, and an activity-dependent exocytosis in excitable cells within secretory granules ("regulated pathway") (Lessmann and Brigadski, 2009). BDNF works locally, either secreted by postsynaptic targets (neurons, muscle fibers and astrocytes) or via an autocrine mechanism (Connor and Dragunow, 1998; Saha et al., 2006). The mature form of BDNF binds to the TrkB with high affinity, and activates intracellular signaling cascades, such as the protein kinase A pathway and ERK1/2 pathway (Lessmann and Brigadski, 2009; Duman and Voleti, 2012). Mature BDNF also binds the p75 neurotrophin receptor, but with much lower affinity; however, its precursor pro-BDNF binds p75 with high affinity and induces apoptosis (Cohen-Cory et al., 2010).

The permissive effect of BDNF on neurite branching is supported by a number of studies (Chakravarthy et al., 2006; Gao et al., 2009; Wang et al., 2015). For example, conditional knockout

of BDNF in adult mice hippocampal neurons led to decreased number and length of dendritic branches (Gao et al., 2009). Localized BDNF production stimulated by treadmill training in rodents, led to enhanced dendritic plasticity and recovery from spinal cord injury (Wang et al., 2015). Truncated TrkB receptors impaired dendritic spine maintenance in primary visual cortex of adult mice (Chakravarthy et al., 2006).

BDNF was also reported to contribute to axon branching, pathfinding and regeneration (Chen et al., 2006; Wilhelm et al., 2012). BDNF acted by increasing the activity of Cdc42 and its downstream effector ADF/cofilin to regulate growth cone motility in retinal neurons (Chen et al., 2006). Axon regeneration in BDNF (-/-) grafts was reduced after tibial nerve injury in mice, while exogenous BDNF reversed this effect and enhanced axon elongation in the BDNF (-/-) graft (Wilhelm et al., 2012). The effects of BDNF on axon elongation are complex. For instance, activation of the adenosine $A_2$ receptor stimulated both axon elongation and neurite branching (Ribeiro et al., 2016). However, only neurite branching was blocked by the extracellular BDNF scavenger TrkB-Fc, whereas axon elongation was not changed. Moreover, patterns of BDNF secretion may affect target cell response (Ji et al., 2010). The work of Ji et al. suggested that activity regulated BDNF secretion leads to transient TrkB activation, which resulted in neurite elongation and mushroom-shaped spine heads in dendrites. Conversely, constitutively secreted BDNF elicited sustained TrkB activation, which in turn stimulated branching.

In the current study, exogenous BDNF increased the number of secondary neurites in HBG3-MNs, while the number of primary neurites was not altered. BDNF also increased total neurite length, which most likely resulted from the increased number of secondary neurites. In contrast, the results of our earlier studies indicate that BoNT/A increases neurite length by stimulating the formation of both primary and secondary neurites. When TrkB-Fc was applied to

the culture of HBG3-MNs in the presence of BoNT/A, the neuritogenic effect of BoNT/A was completely blocked. Given that our data also revealed an up-regulation of BDNF by BoNT/A, the collective findings suggest that BoNT/A may act by inducing BDNF expression and secretion to stimulate secondary neurite formation, while the mechanism of BoNT/A action on primary neurite formations remains unknown. One hypothesis is that other neurotrophic ligands that activate the TrkB receptor may mediate BoNT/A-induced primary neurite formation. Several possibilities exist for activators of TrkB independent of BDNF. Neurotrophin-4 (NT-4) also binds and activates the TrkB receptor (Huang and Reichardt, 2003). NT-4 has been shown to promote neurite outgrowth in geniculate ganglion neurons through the TrkB receptor, with the same affinity but almost 100-fold higher potency than BDNF (Runge et al., 2012). Neurotrophin-3 primarily binds the TrkC receptor with high affinity (Ryden and Ibanez, 1996). Interestingly, it also has a domain that could recognize p75, TrkA and TrkB receptors. In addition, Trk receptors can be transactivated by G-protein coupled receptor (GPCR) ligands (e.g adenosine), although exactly how these two receptors interact to contribute to neurite outgrowth is unclear (Rajagopal et al., 2004). GPCRs have been reported in several studies to promote neurite outgrowth via the cyclic adenosine monophosphate pathway (Aglah et al., 2008; Shelly et al., 2010; Stiles et al., 2014).

ERK1/2 signaling has been shown to be critical for neurite outgrowth in many types of neurons (Perron and Bixby, 1999; Qiu et al., 2004; Harrill et al., 2010). Soluble growth factors (e.g. basic fibroblast growth factor), as well as adhesions molecules (e.g. laminin or N-cadherin) have all been shown to employ ERK1/2 in downstream signaling to induce neurite growth (Perron and Bixby, 1999). In addition, ERK1/2 activation is often a downstream effect of BDNF-TrkB signaling (Duman and Voleti, 2012; Liu et al., 2016). In the current study, inhibition of ERK1/2 activation blocked the BoNT/A induced *bdnf* expression. Since both the ERK1/2 pathway (chapter

3) and BDNF induces secondary neurite formation and they both are downstream effectors of BoNT/A, it can be hypothesized that BoNT/A acts through the ERK1/2 pathway to up-regulate *bdnf* expression and promote secondary neurite outgrowth. The ERK1/2 signaling pathway has been reported to induce *bdnf* expression (Takayama and Ueda, 2005; Matsuoka and Yang, 2012; Okuyama et al., 2012). ERK1/2 could do so by phosphorylating the cyclic AMP-response element binding (CREB) transcription factor (Shaywitz and Greenberg, 1999; Okuyama et al., 2012). Confirmation that BDNF-TrkB signaling induced by BoNT/A to promote neurite outgrowth is mediated through ERK1/2 activation will need further investigation.

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Table 4.1. Summary of neuritogenesis relevant genes in MNs following BoNT/A exposure.

| GENES | SYMBOL | FOLD REGULATION |
|---|---|---|
| **Up-Regulated Genes** | | |
| Protein kinase C, alpha | Prkca | 1.1883 |
| Brain derived neurotrophic factor | Bdnf | 1.9138 |
| Zinc finger protein 91(CNTF) | Zfp91 | 1.2493 |
| Interleukin 2 | Il2 | 2.1942 |
| Leptin | Lep | 3.4979 |
| **Down-Regulated Genes** | | |
| Cyclin-dependent kinase inhibitor 2B | Cdkn2b | -1.3364 |
| Signal transducer and activator of transcription 4 | Stat4 | -1.876 |
| **Related Genes with No Significant Changes** | | |
| Thymoma viral proto-oncogene 1 | Akt1 | -1.0179 |
| Glycogen synthase kinase 3 beta | Gsk3b | -1.0418 |
| Ras homolog gene family, member A | Rhoa | -1.0151 |
| WNT1 inducible signaling pathway protein 1 | Wisp1 | -1.0013 |
| Nitric oxide synthase 2, inducible | Nos2 | 1.2594 |
| Jun oncogene | Jun | 1.1188 |
| Nerve growth factor | Ngf | 1.4288 |
| Glial cell line derived neurotrophic factor | Gdnf | -1.1174 |
| Neurotrophin 3 | Ntf3 | -1.0096 |
| Fibroblast growth factor 2 | Fgf2 | 1.4002 |
| Interleukin 6 | Il6 | 0.412334 |
| Actinin, alpha 1 | Actn1 | -1.1473 |
| Integrin beta 1 (fibronectin receptor beta) | Itgb1 | 1.0373 |

Mouse embryonic MNs were treated with 1 nM of BoNT/A for 12 hours. Microarray analysis was done using categorized array plates. $P<0.1$ for significance.

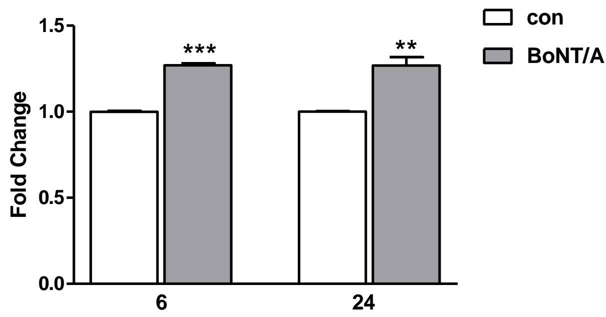


Figure 4.1: Changes in *bdnf* expression in HBG3-MN cultures following BoNT/A exposure. Cells were exposed to 1 nM BoNT/A for 6 and 24 hours. Comparison to control was determined (mean ± S.E.M., n=3) by the $^{\Delta\Delta}C_T$ method using *gapdh* as a house keeping gene.

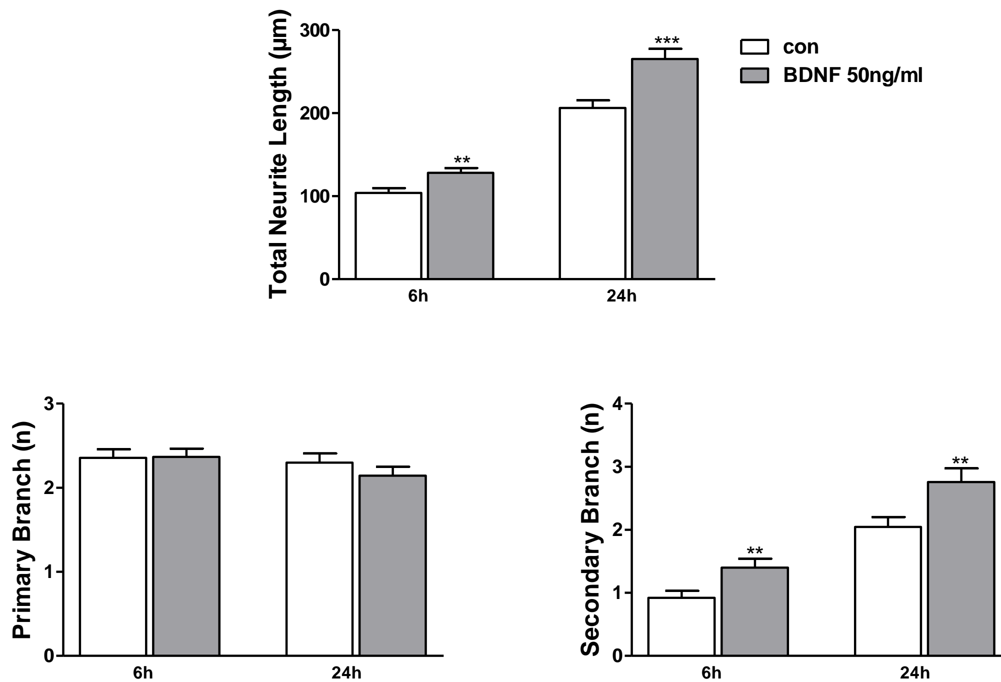


Figure 4.2: Effects of exogenous BDNF on neurite outgrowth. HBG3-MNs were exposed to 50 ng/ml BDNF for 6 and 24 hours. Comparison of total length, primary and secondary neurite numbers (mean ± S.E.M.) to control were made using t-test. $^{**}P<0.01$, $^{***}P<0.001$.

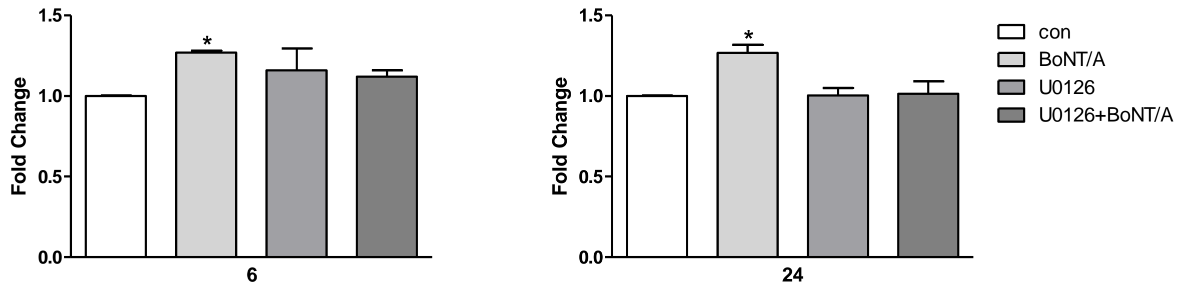


Figure 4.3: Effect of U0126 on *bdnf* expression in HBG3-MN cultures following BoNT/A exposure. Cells were exposed to either 1 nM BoNT/A, or 10 µM U0126, or both for 6 and 24 hours. Comparison to control (mean ± S.E.M., n=3) was determined by the $^{\Delta\Delta}C_T$ method using *gapdh* as a house keeping gene. *$p$<0.05.

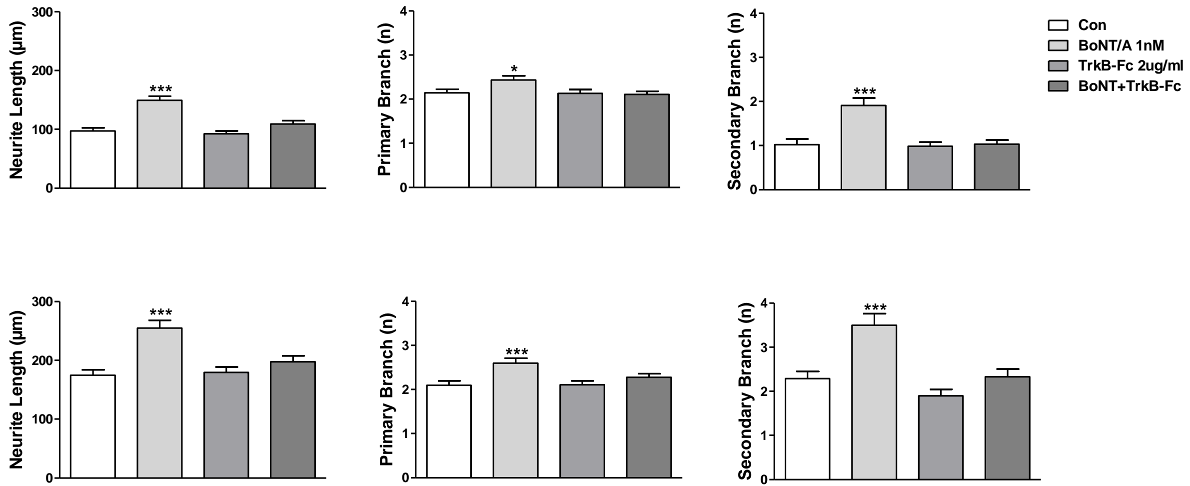


Figure 4.4: Effect of TrkB-Fc on BoNT/A induced neurite outgrowth. HBG3-MNs were exposed to either 1 nM BoNT/A, or 2 µg/ml TrkB-Fc, or both. Upper and lower panels represent exposure for 6 and 24 hours, respectively. Comparison of total length, primary and secondary neurite numbers (mean ± S.E.M.) to control were made using ANOVA. *$P$<0.05, ***$P$<0.001.

# CHAPTER 5

## SUMMARY AND CONCLUSIONS

The overall goals of the studies in this dissertation were to identify the intracellular signaling pathways mediating BoNT/A-induced neurite outgrowth (‘sprouting’) using HBG3-MNs, and identify potential effector molecules involved. The data (chapter 2) first showed that during the basal neurite development of HBG3-MNs in culture, blocking ERK1/2 activation reduced secondary branch formation, while primary neurite formation was not significantly altered. The decrease of total neurite length is likely due directly to the reduced number of secondary neurites. The neurotrophin BDNF enhanced ERK1/2 phosphorylation and the BDNF neurite outgrowth assay confirmed the permissive action of ERK1/2 on secondary, but not primary neurite development in the HBG3-MNs (chapters 2 and 4). This indicates that ERK1/2 activation is responsible for BDNF-induced secondary neurite formation in these cells. The AKT and classical PKC isoform pathways do not have significant effects on basal neurite outgrowth of HBG3-derived MNs, although they were reported to be activated by BDNF (Duman and Voleti, 2012). As has being discussed in previous chapters, additional studies are needed to clarify the activities of AKT and PKC. Approaches include detecting the phosphorylation of AKT and PKC isoforms using western blot, including a wider dose range of PKC inhibitor and including positive control treatments. Current literature on whether BDNF-induced neurite outgrowth is mediated by the ERK1/2 pathway is not conclusive. Some evidence in other neuronal models (e.g. dorsal root ganglion neurons and hippocampal pyramidal neurons) is in agreement with the current findings,

although the neuritogenic effect of BDNF in cochlear spinal ganglion neurons was mediated by AKT and P38 signaling, instead of ERK1/2 (Alonso et al., 2004; Mullen et al., 2012; Liu et al., 2016).

BoNT/A's neuritogenic effect, which is independent of its paralytic action, has been observed for decades, but the neuronal mechanisms remained unresolved. BoNT/A increased primary and secondary branch formation as well as total neurite length in HBG3-MNs (chapter 3), similar to previously published findings for mouse primary MNs (Coffield and Yan, 2009). Immunoblot analysis showed that the phosphorylation of ERK1/2 was enhanced transiently in cultures of HBG3-MNs in response to BoNT/A exposure. Blocking ERK1/2 activation reduced the stimulatory effects of BoNT/A on both primary and secondary neurites, whereas blocking the activity of AKT or classical PKC isoforms at the recommended doses did not. These data are the first to demonstrate the critical role of ERK1/2 signaling in mediating BoNT/A-induced neurite outgrowth. Particularly, BoNT/A enhanced the basal level of ERK1/2 activity in the cultures, which resulted in increased numbers of both primary and secondary neurites, as well as total neurite length. Thus, although activation of the ERK1/2 pathway was not critical for primary neurite formation during basal neurite development (chapter 2), it played a critical role in BoNT/A-induced primary neurite formation, and blocking the ERK1/2 pathway reversed the toxin's stimulated effect (chapter 3). Collectively these data suggest 1) differential regulation of primary and secondary neurite outgrowth during basal development, as well as 2) differential regulation of primary neurite outgrowth under basal and toxin-stimulated conditions.

Initial RT-PCR microarray analyses revealed that *bdnf* expression was elevated in response to BoNT/A exposure in cultured embryonic primary MNs, and subsequent quantitative PCR confirmed the upregulation of *bdnf* in cultures of HBG3-MNs (chapter 4). Inhibition of the ERK1/2

pathway blocked BoNT/A induced *bdnf* upregulation. The BDNF scavenger TrkB-Fc caused nearly complete blockade of the stimulatory effect of BoNT/A on both primary and secondary neurite formation, as well as total neurite length. Taken together with the data from chapters 2 and 3, it is proposed that BoNT/A exposure upregulates *bdnf* expression by activating the ERK1/2 pathway, and the increased production of BDNF stimulates secondary neurite formation only and increases total neurite length. The mechanism for BoNT/A-induced promotion of primary neurite formation, while also mediated by the ERK1/2 pathway, may depend on factors other than BDNF. Interestingly, a recent study disclosed somewhat similar findings (Ribeiro et al., 2016). Instead of using BoNT/A, the adenosine agonist CGS21680 was used to stimulate neurite outgrowth in mature cortical neurons. Activation of the adenosine $A_2$ receptor stimulated both axon elongation and dendritic neurite branching. However, only neurite branching was dependent on BDNF and blocked by the extracellular BDNF scavenger TrkB-Fc, whereas, axon elongation was not. The authors proposed that this differential effect might be related to potential reciprocal interactions between adenosine receptors (frequently coupled to Gs proteins) and TrkB receptors. In the current studies, potential explanations for the different effect on primary neurite between BDNF and TrkB-Fc are: 1) TrkB activation by other neurotrophins, or 2) transactivation of TrkB by other signaling cascades. Further experiments are needed to test out the hypotheses.

The findings in the current study demonstrate that neurite development of HBG3-MNs in culture at early stages involves the ERK1/2 pathway, although BDNF-induced neuritogenesis has been reported to be mediated by ERK1/2, PI3K-AKT, and PLC pathways, as mentioned earlier in this dissertation (Duman and Voleti, 2012; Ribeiro et al., 2016). In addition, the critical role of ERK1/2 pathway activation in BoNT/A-induced neurite outgrowth in HBG3-MNs can be summarized as the following: 1) ERK1/2 mediated basal development of secondary neurites is

further enhanced by BoNT/A; 2) ERK1/2 mediated primary neurite formation is induced by BoNT/A; 3) ERK1/2 mediates up-regulation of *bdnf* when stimulated by BoNT/A (Fig. 5.1). How this pathway is tightly regulated and delivers various cellular responses in this scenario remain to be elucidated. Moreover, immunoblots indicate that the ERK2 (42 kDa) is dominant in HBG3-MNs at early stages, especially within 24 hour, compared to the band density of ERK1 (44 kDa). Stimulation by either BDNF or BoNT/A enhanced ERK2, while changes in ERK1 was not obvious. The concept of functional redundancy between ERK1 and ERK2 is currently accepted, although some isoform specific functions could not be excluded as has been reported in a few knock-down studies (Fremin et al., 2015; Busca et al., 2016).

One thing worth noting, total neurite length as measured in HBG3-MNs does not equate to neurite elongation. Neurite elongation more likely defines the length of an axon, which can be difficult to differentiate from other neurites without staining for specific markers (e.g. Tau1), especially for neurons at an early age (Ribeiro et al., 2016). The elongation of axons requires more cytoskeletal motility, which is regulated by growth cones at the tip of axons in response to permissive/repulsive growth cues in the environment. Although the mechanisms for elongation are not fully elucidated, current literature suggest that such cytoskeletal dynamics can be triggered by calcium signaling, and cAMP is most likely to be the key player in its regulation (Aglah et al., 2008; Shelly et al., 2010; Kalil et al., 2011). It will be interesting to examine the effect of BoNT/A on axon elongation as well as the involvement of cAMP signaling in this process.

As mentioned in chapter 1, I used HBG3-MNs as our research model in this dissertation. This model has many advantages over primary neurons and hybrid neuronal cell lines. The mixed population derived from stem cells might also provide support to MNs giving the evidence of GFAP+ cells in the culture, so that MNs can grow healthy and isolated without clustering. This

allows us to perform better neurite outgrowth assays with a large samples size and few tangled/overlapped neurites. For other methods used in this dissertation, such as immunoblotting and q-PCR, the interpretation of the results needs to take into consideration the mixed population of cells with GFP+ MNs. Although not characterized in the literature, our preliminary data suggested the majority of those non-GFP+ cells were GFAP+, which could be astrocytes and/or neuronal precursors that give rise to neurons and oligodendrocytes (Menn et al., 2006). Currently no study has reported that BoNT/A affects astrocytes, although one study stated that BoNT/B and /F entered cultured astrocytes and partially cleaved cellubrevin, a synaptobrevin homologue in nonneuronal cells (Verderio et al., 1999). Although astrocytes normally are present in the central nervous system, it would be ideal to confirm the results of these studies herein by performing immunoblot and q-PCR assays using pure HBG3-MNs to rule out potential confounding factors from the mixed population. Fluorescence activated cell sorting is one approach and have been used with success in these cells (Kiris et al., 2011).

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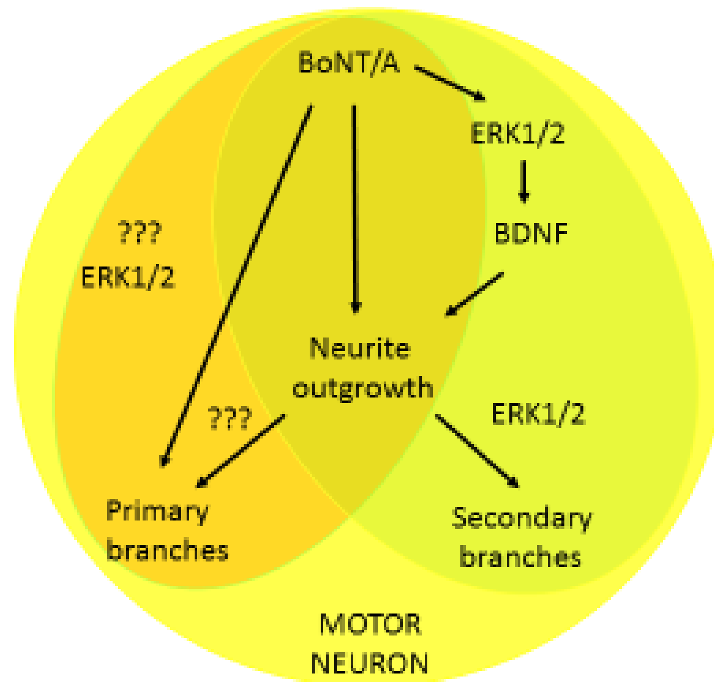


Figure 5.1: Schematic diagram depicting proposed mechanisms of BoNT/A-induced neurite outgrowth.