Characterization of Pseudophosphatase MK-STYX-Induced Neuronal Differentiation in PC12 Cells

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Characterization of Pseudophosphatase MK-STYX-Induced Neuronal Differentiation in PC12 Cells

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in the Department of Biology from The College of William & Mary

By

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ABSTRACT

MK-STYX [MAPK (mitogen activated protein kinase) phosphoserine/threonine/tyrosine binding protein] is a pseudophosphatase member of the MAPK phosphatase family. Though structurally related to the MAPK phosphatases, MK-STYX lacks the nucleophilic cysteine and histidine residues essential for catalysis. Despite its lack of catalytic activity, MK-STYX maintains its ability to bind to phosphorylated proteins, but not dephosphorylating them. This thesis focuses on further characterizing the role of MK-STYX in PC12 neuronal differentiation. The PC12 cell line is widely used as a model to study neuronal differentiation. Our previous data demonstrated that MK-STYX induces neuronal differentiation in PC12 cells. The results presented here also show that MK-STYX induces neuronal differentiation in PC12 cells. We further investigated whether MK-STYX induced a unique branching pattern in PC12 cells when they differentiate. In the presence and absence of nerve growth factor (NGF), both primary and secondary neurite distributions are changed, suggesting that MK-STYX changes neurite branching patterns. To investigate what caused this pattern, we turned to the cytoskeleton. Actin polymerization drives the protrusion of lamellipodia and filopodia in growth cones, while tubulin is responsible for making up the ‘core’ of a developing neurite. In the presence of NGF, PC12 cells overexpressing MK-STYX caused an increase in actin protrusions commonly associated with growth cones, dendritic spines, and future dendritic branches. In addition, MK-STYX induced neurites exhibit presynaptic and post-synaptic qualities, as shown through immunostaining with anti-Tau and MAP2 antibodies, respectively. Cofilin, an actin binding and severing protein, has been shown to play a role in regulating neurite outgrowth and branching. MK-STYX further regulates actin dynamics, and thus branching through a possible temporal regulation of cofilin phosphorylation and dephosphorylation. A transient decrease in cofilin phosphorylation at 72 hours of NGF stimulation allows MK-STYX-induced neurites to branch. This strongly supports a model in which the pseudophosphatase MK-STYX has a critical role as a regulator in PC12 neuronal differentiation and morphology.
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INTRODUCTION

PC12 Cells as a Neuronal Model

The rat pheochromocytoma (PC12) cell line is derived from a transplantable rat pheochromocytoma of the adrenal medulla. These cells originate from the embryonic neural crest, and have a mixture of eosinophilic and neuroblastic cells (Greene and Tischler, 1976). This neuroblastic origin gives PC12s the ability to differentiate into neuron-like cells. Despite not being a primary neuronal cell line, PC12 cells have been used as a model system for neuronal development, neuronal signal transduction, and neurosecretion. PC12 cells are capable of differentiating into sympathetic-like neurons when exposed to neurotrophins, such as nerve growth factor (NGF), and other signaling molecules, such as cAMP (cyclic AMP) (Young et al., 1994). Exposure to neurotrophins causes a cessation of normal progression through the cell cycle. However, PC12 cell survival is not dependent upon a continuous exposure to NGF (Fujita et al., 1989; Greene and Tischler, 1976). This cessation of normal cell cycle progression is elicited by the binding of NGF to the neurotrophic tyrosine kinase receptors, with a majority of NGF binding to TrkA (Weismann and de Vos, 2001). In addition, persistent TrkA activity is required in order to activate and maintain gene expression in differentiating PC12 cells (Chang et al., 2003). This differential response is in part due to differences in MAPK (mitogen activated protein kinase) activation. Stimulation with NGF causes sustained activation of the MAPK pathway, while stimulation with epidermal growth factor (EGF) causes a brief transient activation of the MAPK pathway (Traverse et al., 1992; Traverse et al., 1994). Thus, sustained MAPK phosphorylation induced by NGF leads to neuronal differentiation in PC12 cells (Figure 1), while rapid MAPK dephosphorylation induced by EGF leads to proliferation of cells.
**Figure 1. NGF induces neuronal differentiation in PC12 cells.**

Representative inverted phase micrograph of an non-transfected PC12 cell before and after stimulation with NGF, a neurotrophin. Images were captured prior to NGF stimulation, and then stimulated with 100 ng/ml NGF for three days as described in the methods. Due to a sustained activation of the MAPK cascade induced by NGF stimulation, transcription of neuronal genes commences, leading to neurite outgrowths.

**Kinases in Neuronal Development**

Three signal transduction pathways commonly associated with neuronal differentiation are the MAPK cascade, the phosphatidylinositol 3-kinase cascade (PI3K), and the phospholipase C cascade. The former two regulate neuronal development, while the latter is responsible for neurotrophin release (Hausott et al., 2009).

The MAPK cascade is the best characterized of the three. This signal transduction cascade is caused by phosphorylation cascades of MAPK kinase kinase (Raf-1), MAPK kinase
(MEK), and MAPK (ERK 1/2). Ras GTPase activates MEKK, MEK, and MAPK to induce either proliferation, differentiation, or apoptosis depending upon the signal response (Rojas et al., 2012). In neuronal cells, a sustained activation of the MAPK cascade, particularly the Extracellular regulated kinases (ERK1/2), leads to the transcription of genes required for neuronal development. ERK1/2 is activated by MEK, which phosphorylates its’ threonine and tyrosine residues (Sweatt, 2001). A transient activation of ERK1/2 leads to cell proliferation, while a sustained activation induces neuronal differentiation. This is best demonstrated by stimulation with NGF or EGF. NGF stimulation induces sustained activation of ERK1/2 and leads to PC12 differentiation, while EGF stimulation causes a transient activation of ERK1/2, resulting in proliferation (Kao et al., 2001; Traverse et. al., 1992; Traverse et. al., 1994).

Phosphatases are able to temporally regulate the rate and duration of ERK1/2 activation by removal of a phosphate group, thus causing its inactivation. Since phosphatases are able to regulate the rate and duration of these signaling cascades, it is important to understand their effects in neuronal differentiation.

Protein Tyrosine Phosphatases in Neuronal Development

Phosphatases, in contrast to the kinases, are able to catalyze the removal of a phosphate group from their substrate, thus regulating phosphorylation cascades that are initiated by the kinases. The protein tyrosine phosphatases (PTPs) are catalyzed by the signature motif: His-Cys-X5-Arg (HCX5R) (Tonks, 1989). The classical PTPs catalyze the removal of phosphate groups from tyrosine residues. Another family within the PTP superfamily are the dual-specificity phosphatases, which can remove phosphate groups from serine/threonine as well as tyrosine residues (Tonks, 1989). Two classical PTPs, SHP-1 (Src homology region 2 domain-containing phosphatase-1) and SHP-2 play a role in neuronal differentiation. SHP-1 negatively regulates
neuronal survival, while SHP-2 induces neuronal differentiation in PC12 cells (Huang et al., 2012; Zhang, 2002).

Of particular importance in neuronal development are the receptor PTPs (RPTPs). The RPTPs are grouped into eight subfamilies, and all members of the RPTP contain at least one cytoplasmic tyrosine phosphatase domain (Ensslen-Craig and Brady-Kalnay, 2004). Regulation of RPTPs may be accomplished via a phosphatase-dependent or phosphatase independent method. For example, preventing phosphatase activity via phosphatase inhibitors prevents RPTP mediated growth cone development, preventing axon growth and guidance, and also inhibits proper dendritic arborization (Sun et al., 2000; Tanaka et al., 2003). Here, arborization is defined as the branching of a dendrite into its’ tree-like morphology. RPTPs have a major role in axon guidance such as promoting target recognition, acting as inhibitory guidance cues, and regulating synapse formation (Ensslen-Craig and Brady-Kalnay, 2004, Sun et al., 2000). Beyond RPTPs there are cytoplasmic PTPs, such as PTP1, that have a role in neuronal development (Sahin et al., 1995). Furthermore, some of these cytoplasmic PTPs are categorized as pseudoenzymes.

Pseudoenzymes

Pseudoenzymes are proteins that have amino acid substitutions in their catalytic motif that render them inactive; however, they still retain the capacity to bind to phosphorylated residues. This classification is split further into the pseudokinases and the pseudophosphatases. The former being unable to catalyze the addition of a phosphate group to its substrate, and the latter unable to catalyze phosphate group removal from its phosphorylated substrate. It is thought that pseudoenzymes evolved to carry out different functions through different domains, and there are four primary modes of action by which pseudokinases and pseudophosphatases can exert a regulatory effect. Pseudokinases and pseudophosphatases can serve as modulators, competitors,
spatial anchors, and signal integrators (Reiterer et al., 2014) (Figure 2). As such, pseudoenzymes have the potential to be regulators of disease pathogenesis. For example, the His domain containing protein tyrosine phosphatase (HD-PTP) is part of a gene cluster that is often missing in human cancers, suggesting that it might play a role in tumor suppression (Hao et al., 2014). The pseudokinase KSR1 (kinase suppressor of Ras 1), which has modulatory and integratory effects on the MAPK cascade, also plays a role in cancer by regulating cell invasion (Lin et al., 2011). With the revelation that pseudoenzymes can be implicated in human disease, more and more research is being done to characterize these enzymes.

HD-PTP is a pseudophosphatase that is involved in the transport of ubiquitinated proteins in the lysosomal system (Doyotte et al., 2008). This pseudophosphatase serves as an anchor for the trapping of substrates, as well as serving as a scaffold to allow signal integration. HD-PTP binds to the epidermal growth factor receptor (EGFR) when it is phosphorylated. Meanwhile, it also associates with STAM2 (signal transducing adaptor molecule 2), a subunit of ESCRT-0 (endosomal sorting complexes required for transport-0). HD-PTP also recruits ESCRT-III, which competes with STAM2 for binding. When ESCRT-III binds, ubiquitin specific peptidase 8 (USP8) is recruited to the HD-PTP/ESCRT-III complex (Ali et al., 2013). USP8 can then deubiquitnate EGFR. Deubiquitination of EGFR allows it to be internalized into the lumen. HD-PTP is responsible for the integration of signals via a pseudophosphatase scaffold, and spatially organizing both the EGFR substrate and its binding partners, allowing for this localization to occur.

The myotubularin-related phosphatases (MTMR) are members of the PTP family that are capable of depophosphorylating the phosphatidylinositolos (Reiterer et al., 2014). MTMR9, a catalytically inactive MTMR is shown to act as a modulator of the catalytically active MTMRs.
MTMR9 can form dimers with the active MTMR6 and MTMR8 thus regulating their activity. When MTMR9 complexes with MTMR6, stress-induced apoptosis is inhibited by changing the substrate specificity of MTMR6 (Zou et al., 2012).

The prototypical pseudophosphatase, STYX (serine threonine tyrosine interacting protein) has a glycine residue in the place of the catalytic cysteine residue (Wishart et al., 1995). During neuronal differentiation, STYX competes with MKP-2 to bind to ERK1/2, reducing its activation and preventing PC12 cells from differentiating (Reiterer et al., 2013). Given that STYX is not related to the MAP kinase phosphatases (MKPs), which act antagonistically to the MAP kinases, its’ effects on PC12 differentiation are striking. MK-STYX, in contrast to STYX, is a pseudophosphatase in the MKP family (Wishart et al., 1995). The causative substitutions rendering MK-STYX catalytically inactive are phenylalanine and serine substitutions in the place of the histidine and catalytic cysteine residues in the protein tyrosine phosphatase (PTP) signature motif (Wishart and Dixon, 1998). In the N-terminal domain, MK-STYX has a cdc homology 25 (CH2) domain, responsible for specificity in MKP-MAPK interactions (Wishart and Dixon, 1998). Given that MK-STYX is a structural homolog to the active MKPs, one would expect that its catalytic activity could be restored. Site directed mutagenesis converting the phenylalanine and serine residues back to the histidine and catalytic cysteine residues generates a catalytically active mutant of MK-STYX, MK-STYX$_{active}$ (Hinton et al., 2010). Previously, little was known about MK-STYX other than it is highly expressed in Ewing’s sarcoma family tumors (Siligan et al., 2005). However, determining the interacting partners of MK-STYX has shed more light on its elusive functions. Earlier studies conducted have shown that MK-STYX binds G3BP (Ras-GTPase activating protein SH3 domain binding protein-1), which has a role in stress granule assembly and the Ras signaling pathway (Hinton et al., 2010). It was then shown that
MK-STYX inhibits stress granule formation independent of phosphorylation at Ser149, a residue whose phosphorylation is critical for stress granule formation (Hinton et al., 2010; Barr et al., 2013; Tourriere et al., 2003). Since G3BP is also implicated in the Rho signaling pathways, MK-STYX may be a regulator of this pathway (Zhang et al., 2007). G3BP also interacts with other molecules, such as tau mRNA, and has been shown to be a player in differentiation of neuronal cells (Atlas et al., 2004; Martin et al., 2013). Given that MK-STYX has been shown by our lab to have a role in neuronal differentiation in PC12 cells, we sought to further characterize the effects of MK-STYX induced neuronal differentiation.

Figure 2. Possible functions of pseudoenzymes.
Despite lacking catalytic activity, pseudoenzymes are key regulators of important signaling pathways. Since they lack catalytic activity, they must exert their regulatory effects in a different manner than their active homologs. (A) Pseudoenzymes can serve as modulators by dimerizing with active kinases/phosphatases, either inhibiting or promoting their activity. (B) Pseudoenzymes can serve as competitors and bind to the substrates of active kinases or phosphatases, preventing their access. (C) Pseudoenzymes may serve as a localization anchor, and can trap a substrate to a particular cellular locale. In this example, the pseudoenzyme localizes the substrate to the nucleus, and will not be exported unless the enzyme/substrate anchor complex is dissociated. (D) Pseudoenzymes can serve as integrators, and can bring together signals from multiple sources.

Rho GTPases in neuronal development

The Rho GTPase family belongs to the Ras superfamily of guanine nucleotide binding proteins. The Rho GTPases serve as a molecular switch by switching between a GTP (guanosine triphosphate)-bound active conformation and a GDP (guanosine diphosphate)-bound inactive conformation. Guanine nucleotide exchange factors (GEFs) exchange GDP for GTP, thus activating the GTPase. Meanwhile, GTPase activating proteins (GAPs) mediate the hydrolysis of GTP to GDP, thus serving as a negative regulator of GTPase activity, while guanine nucleotide dissociation inhibitors (GDIs) accomplish this by preventing the exchange of GDP for GTP (Govek et al., 2005). GDI dissociation allows Rho GTPases to localize to the membrane, allowing for their subsequent activation (Stankiewicz and Linseman, 2014).

The most extensively studied members are RhoA, Rac1, and Cdc42. Collectively, the Rho GTPases are responsible for the organization of the actin and microtubule cytoskeleton. As such, the Rho GTPases play a role in growth cone dynamics, dendrite formation, axonal growth,
and pathfinding. In hippocampal neurons exposed to *Clostridium difficile* Toxin B, an inhibitor of RhoA, Rac, and Cdc42, there is a loss of F-actin accompanied by the formation of protrusions resembling axons (Braadke and Dotti, 1999). Rac/Cdc42 and Rho have an antagonistic relationship with each other when establishing neuronal morphology, Rac/Cdc42 positively regulate neuronal differentiation, which Rho negatively regulates neuronal differentiation. In neuroblastoma cells, overexpression of Rac or Cdc42 enhances neurite outgrowth and growth cone formation, while overexpression of RhoA leads to growth cone collapse and neurite retraction (Kozma et. al., 1997). In addition, RhoA acts upon several downstream effectors, such as coflin, LIM domain kinases (LIMK), and myosin light chain (MLC) responsible for cytoskeleton reorganization (Figure 3).

In order for induction of neuronal differentiation in PC12 cells to occur, RhoA must be inactivated (Jeon et al., 2010). PC12 cells commit to the neuronal phenotype within 24-48 hours, corresponding to minimal levels of RhoA activity (Jeon et. al., 2010). Recently, we showed that MK-STYX reduces RhoA activation in PC12 cells (Flowers et. al., 2014). Throughout time-point stimulation, levels of active RhoA remained low in cells transfected with MK-STYX, while there was a significant increase in the amount of active RhoA in control cells or cells expressing the active mutant. In cells expressing the catalytically active mutant, MK-STYX<sub>active</sub>, RhoA activation is significantly upregulated compared to the control, preventing these cells from differentiating upon exposure to NGF (Flowers et. al., 2014). However, it is not known how MK-STYX mediates this inactivation of RhoA. MK-STYX may interact with low molecular weight protein tyrosine phosphatase (LMW-PTP) or the GAPs and GEFs to inactivate RhoA and induce neuronal differentiation. Alternatively, MK-STYX could directly interact with RhoA, preventing localization to the membrane and subsequent activation (Figure 4).
Given that the Rho GTPases have an important role in neuronal development, dysregulation of these GTPases may be a causative factor in some neurodegenerative diseases. In amyotrophic lateral sclerosis (ALS), Rho-associated kinase (ROCK), a downstream effector of RhoA, has increased activity in mouse models of ALS (Tonges et al., 2014). In Alzheimer’s disease, activation of RhoA leads to an increase in the production of toxic amyloid-beta fragments (Zhou et al., 2003). Furthermore, an increase in Rho activation may be the culprit for neurite retraction and amyloid-beta accumulation seen in the brains of Alzheimer’s patients. Thus, MK-STYX may have a therapeutic role in diseases caused by aberrant RhoA activation.

**Figure 3. RhoA regulation of downstream effectors.**

GTP bound RhoA causes activation of Rho associated kinase (ROCK). ROCK phosphorylates myosin light chain (MLC), while inhibiting myosin light chain phosphatase (MLCP). Phosphorylated MLC results in actin contraction. Therefore, MLC would not be phosphorylated...
when MK-STYX is present. ROCK also activates LIMK, which phosphorylates cofilin, an actin binding protein. Phosphorylated cofilin is inactive, and causes actin filament stability and persistence of neurites. Dephosphorylation induced by chronophin or slingshot activates cofilin’s role as an actin severing protein, allowing for cytoskeletal reorganization and neurite branching.

Figure 4. Proposed means of RhoA inactivation by MK-STYX.

In order to decrease RhoA activation, MK-STYX may interact with LMW-PTP. Dephosphorylation of p190GAP by LMW-PTP inhibits RhoA activity. MK-STYX may also increase the activity of p190GAP, which catalyzes the hydrolysis of GTP to GDP, turning RhoA off. MK-STYX may also inhibit the RhoGEF, preventing RhoGEF from exchange GDP to GTP,
preventing RhoA from reactivating. Finally, MK-STYX could interact directly with RhoA, preventing localization to the membrane that is necessary for subsequent interaction, or by preventing RhoGEFs and p190GAP from acting upon GTP-bound RhoA.

Cytoskeletal Regulation in Neuronal Morphology

Several downstream effectors of RhoA, such as ROCK, LIMK, and coflin, play a role in the regulation of the cytoskeleton, particularly actin elements. The actin cytoskeleton plays a major role in the morphological development of neurons, from their conception to maturation. Neurons extend axons and dendrites, which are led at the end by a growth cone, composed of finger-like lamellipodia and veil-like filopodia. In rapidly extending and retracting neurites, filopodia are composed of bundled F-actin fibers, while lamellipodia have a cross-linked network of actin. When an axon reaches its target, the growth cones are converted into presynaptic terminals (Luo, 2002). Upon contact with an axon, dendrites form a postsynaptic terminal, which combines with the presynaptic terminal of a mature axon to develop a synapse. In some cases actin rich protrusions, called dendritic spines, develop along the length of the dendrite, also play a role in synaptic activity. However, the process by which axons and dendrites are initiated are quite different.

In primary hippocampal neurons, establishment of the axon begins when the cell sends out minor processes, which are small, underdeveloped neurites. One of these minor processes will start to grow much more rapidly than the other, ultimately becoming the axon (Dotti et al., 1998). In the growth cone of an a extending axon, actin monomers at the leading edge cause filopodia and lamellipodia to extend; however, F-actin flowing away from the leading edge can cause retraction (Mallavarapu & Mitchison, 1999). When a growth cone is extending towards a
target, retrograde F-actin flow is no longer isotropic, and decreases in the direction of growth cone extension (Lin & Forscher, 1995). This dynamic process of extension and retraction allows a developing axon to reach its target. Complementary to the actin network, microtubules play a role in the formation of axons. During axon formation, stabilization of microtubules allows for the dynamic, stable end to protrude at the edge of a developing neurite, promoting axon formation.

In contrast, dendritic development is much more complex. In most regards, cytoskeletal regulation of the growth, guidance, and branching of axons is the same for dendrites, and many of the molecules that regulate dendritic growth have similar roles in axons (Scott & Luo, 2001). The most notable feature of dendrites are their branching patterns. Unlike axons, branching occurs from the shaft of the dendrites rather than splitting of the growth cone at the leading edge of the developing dendrite. Further branching of the dendritic tree occurs by stabilization of these branches, which allows for the addition of subsequent branch additions. This process repeats until the proper dendritic arbor is achieved for that cell type. This stabilization is mediated by the filopodia. Filopodia stabilization during early dendritic development results in the formation of dendritic branches, while stabilization late in development leads to the formation of dendritic spines (Dailey & Smith, 1996).

Mechanisms of Dendrite Growth

In order to reach maturation, a developing neuron undergoes several stages. After cells first begin to differentiate into neurons, they localize to their appropriate place within the developing nervous system. Then, depending on the cell type, neurons shape their axons and dendrites into patterns that are characteristic of their respective cell type. Once axonal and dendritic elaboration has concluded synapses are formed, which are connections between
different neurons. In a majority of cell types, dendrites have the majority of synaptic contacts; as such, dendrites are responsible for determining the patterning of synaptic connections between neurons (Purves et al., 1986). Thus, proper dendrite morphology is essential for the development of a ‘healthy’ nervous system. Consequently, errors in the regulation of the dendritic cytoskeleton can have negative effects on the formation and functionality of synapses (Newey et al., 2004). For example, X-linked mental retardation has been associated with improper dendritic arborization, and in Down’s syndrome, dendritic branching is less complex and continues to become more simplified as a patient ages (Fiala et al., 2002; Kaufmann and Moser, 2000; Takashima et al, 1994). Given the biological consequences that aberrant dendritic morphology entails, regulation of normal dendritic development is important.

Initially, dendritic development was thought to be the result of an alternating process of extending and branching (McAllister, 2000). However, the formation of dendrites is a much more dynamic process than initially thought. Dendritic development is slow in the beginning, but as growth rates increase, there is a temporary overproduction of dendrites (Koester and O’Leary, 1992). In actuality, dendrites extend and retract throughout the course of development, before solidifying on the pattern common to their respective cell type. The final form of a cell’s dendritic arbor is dependent upon not just intrinsic patterns common to that cell type, but also on environmental cues (McAllister, 2000).

There are two broad groups of molecular signals that regulate dendritic growth: extracellular and intracellular signals. Many of the extracellular molecules that regulate dendritic growth also have a role in axon guidance and synapse formation, such as the secreted protein semaphorins, which act as axonal growth cone guidance molecules, notch signaling, and the neurotrophins. The most widely studied of these extracellular molecules are the neurotrophins.
This group of signaling molecules contains four functionally related proteins: NGF, brain-derived neurotrophic factor (BDNF) neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (McAllister, 2000). Like NGF, the other three neurotrophins also work via binding to Trk receptors, as well as p75, which has low-affinity for most neurotrophins. The first experiments that demonstrated that neurotrophins have a role in regulating dendritic growth showed that NGF treatment for two weeks caused an increase in dendritic arborization in neonatal rats (Snider, 1988; Ruit et. al, 1990).

Many intracellular molecules that regulate axonal growth and development are also responsible for dendritic growth, however, for dendrites, many of these signaling molecules are regulated by synaptic activity. Some of the key players in intracellular regulation of dendritic growth are the microtubule-associated proteins (MAPs), the GTPases as described earlier, and dendritic mRNAs. Many of the intracellular molecules that regulate dendritic growth act upon the cytoskeleton, composed of actin microfilaments and tubulin microtubules in dendrites. During development, microtubules are transported to the growing dendrite by the structural gene for phosphatidylserine synthase, CHO1, and the mitotic kinesin-like protein 1 (MKLP1), allowing for their polymerization (Sharp et. al., 1997). During polymerization microtubules are unstable, and may not persist long in the cell. However, capping the growing end of the microtubule polymer with MAP2, a MAP located primarily in dendrites, allows the growing microtubule to become much more stable (Matus, 1988). The MAP proteins are therefore critical in mediating dendritic growth by regulating filament stability, allowing for the persistence of dendrites.

Thesis Objectives
Prior research conducted in our lab shows that MK-STYX induces neurite-like outgrowths in PC12 cells. Given that MK-STYX decreases RhoA activation, MK-STYX might have an effect on downstream effectors of RhoA, potentially regulating cytoskeletal dynamics. This thesis investigated the role MK-STYX has in PC12 cell neuronal differentiation. The specific aims were as follows:

1.) Does MK-STYX affect the branching pattern of PC12 cells?
2.) Does MK-STYX affect cytoskeletal dynamics during neuronal differentiation?
3.) Does MK-STYX affect the downstream effectors of RhoA?
METHODS

Plasmids

pMT2-FLAG-MK-STYX-FLAG and pMT2-FLAG-MK-STYX\textsubscript{active}-FLAG were generated as described by Hinton et al (2010). The terminal ends of MK-STYX were flanked by the FLAG epitope for MK-STYX detection, and sequence fidelity was determined by sequencing.

Cell culture

All experiments used rat pheochromocytoma PC12 cells (ATCC). PC12 cells were grown and maintained at 37°C and 5% CO\textsubscript{2} in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen). Medium was supplemented with 10% horse serum (Invitrogen) and 5% fetal bovine serum (Invitrogen). Cells were maintained with 15 ml medium per 75 cm\textsuperscript{2} flask (Thermo Scientific), 5 ml per 15 cm\textsuperscript{2} flask (Fisher), 5 ml per 60 mm plate (Thermo Scientific), or 3 ml per well in six well plates (Thermo Scientific). Culture medium was changed every other day, and cells were seeded at a lower confluency by manual trituration via repeated pipetting with a 5 ml pipette once a week.

Transfection

For transient transfections, pMT2 control vector, pMT2-FLAG-MK-STYX-FLAG, pMT2-FLAG-MK-STYX\textsubscript{active}, or pEGFP were used. Twelve to eighteen hours post seeding, PC12 cells were transiently transfected using Lipofectamine 2000 reagent (Invitrogen). Cells were transfected using 2 µl of Lipofectamine 2000 reagent per 1 µg DNA. Transfection mixtures
were prepared using serum-free Opti-MEM (Invitrogen). Cells were incubated with transfection reagents for four to six hours before the medium was removed and replaced with fresh medium.

NGF Stimulation

Twenty-four hours post-transfection, PC12 cells were serum starved in DMEM supplemented with 0.1% fetal bovine serum for eight to twelve hours. PC12 cells were then stimulated with 100 ng/ml of b-NGF (Prospec) throughout experiments.

Time-dependent NGF Stimulation

PC12 cells were transfected, serum starved, and stimulated with NGF as described for time points consisting of 0 minutes, 3 minutes, 5 minutes, 12 minutes, 30 minutes, 24 hours, 48 hours, or 72 hours. Cells were lysed at the indicated time points.

Cell Lysis

PC12 cells were lysed in lysis buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 10% glycerol, 10 mM NaF, 1% Nonidet P-40 alternative [Calbiochem] and protease inhibitor tablets [Rochel]). Cells were rinsed with 10 ml of cold 1X D-PBS. Cells were lysed with lysis buffer for five minutes on ice. Protein concentrations were quantified by NanoDrop. Lysates were stored at -20°C, or snap-frozen with liquid nitrogen and stored at -80°C (longer storage) for immunoblotting analysis.

Immunoblotting
Lysates were sonicated and centrifuged at 14,000 rpm at 4°C for 10 minutes. Protein concentration was determined using NanoDrop quantification. Lysates were diluted to obtain samples of 30 µg or 40 µg of protein with 6X Laemmli sample loading buffer and 2 µl of dithiothreitol (DTT) to obtain a final volume of 20 µl. Lysates were denatured by boiling at 100°C for 5 minutes. Lysates were resolved on 10 or 12% SDS-PAGE gels for 1 hour at 120 volts. Protein gels were transferred to a PVDF membrane (GE Healthcare) using iBlot dry blotting system (Invitrogen). Membranes were immunoblotted with anti-FLAG (Sigma) to detect MK-STYX, anti-β-tubulin (Thermo Scientific) for loading control, cofillin (Cell Signaling), phospho-cofilin (Cell signaling), chronophin (Cell Signaling), or RhoA (Cytoskeleton Inc) in 5% milk 1X TTBS (Tween 20 Tris Saline Buffer). Membranes were probed with a secondary HRP-conjugated antibody, and protein bands were detected with enhanced chemiluminescence plus (GE Healthcare) and analyzed by autoradiography. When warranted, membranes were stripped with mild stripping buffer (200 mM glycine, 3.5 mM SDS, 1% Tween 20 [Abcam]) and reprobed. PKPS (BioRad) was used as a molecular weight marker.

MK-STYX Knockdown

Control or MK-STYX specific shRNA expression plasmids (Qiagen) were transfected into PC12 cells for 24 hour. Knockdowns were validated by Real-Time PCR (Flowers et al., 2014). 24 hour post-transfection cells were stimulated or not with 100 ng/ml NGF, lysed, and used for immunoblotting.

Fluorescence Microscopy
For live imaging, cells were plated at a confluency of $2.5 \times 10^4$ cells per well. Live imaging of PC12 cells was conducted with phase and fluorescence microscopy using Nikon Eclipse Ti inverted fluorescence microscopy.

Quantification of Primary and Secondary Neurite Distributions

To quantify neurite branching, PC12 cells were plated at a confluency of $1 \times 10^5$ cells in a 10 cm plate. Plates were transfected with 2µg of pMT2 or MK-STYX as describe above. 24 hours post transfection, cells were serum starved as described and stimulated with 100 ng/ml NGF for 3 days.

To score for changes in branching distribution between the groups, only cells that had neurites at least 20 µm in length were scored. Length measurements were made using NIS-Elements BR 3.10 software (Nikon). Primary neurites were classified as those neurites at least 20 µm in length which originated from the body of the cell. For each cell meeting these qualifications, the number of primary extensions from the cell body were counted and scored. Then, using the same cell, the presence of secondary branches was scored. Here, we defined a secondary branch as any bifurcation at least 20µm in length along a primary neurite. Thus, each differentiated cell was scored for the presence of primary neurites, and then secondary branches.

Immunostaining

For immunostaining experiments, PC12 cells were seeded at $2.0 \times 10^4$ cells on type I collagen-coated coverslips (Neuvitro) to promote PC12 cell adhesion. Cells were transfected and stimulated with 100 ng/ml NGF for three days as described. Cells were prefixed to preserve cell structure integrity for 2 minutes in 4.0% formaldehyde by adding an equivalent volume (3 ml) of
4.0% formaldehyde to the culture medium in the six wells for a final concentration of 2.0% formaldehyde. Following prefixation, cells were fixed in 3.7% formaldehyde for 8-10 minutes. Cells were washed, then permeabilized by immersion in 0.1% Triton-X-100 for 5 minutes. For actin/tubulin staining, cells were incubated in rhodamine-conjugated phalloidin (1:25) (a kind gift provided by Dr. Shakes, Life Technologies) for 1.5 hours in the dark, and then incubated in β-tubulin-FITC (1:150) for 1 hour in the dark. A detailed protocol of the staining procedure is included in the appendix. For MAP2/Tau staining experiments, cells were incubated with MAP2 (Cell signaling) (1:100, anti-rabbit) and Tau (Pierce) (1:150, anti-mouse) antibodies for 1 hour at room temperature in a humidified chamber. Cells were washed, then incubated with anti-mouse Cy3 (Invitrogen) (1:200) and anti-rabbit Cy5 (Invitrogen) (1:100) for 45 minutes in the dark. Cells were washed, then mounted in Fluoro-gel with DAPI in order to stain DNA. For co-localization experiments, cells were incubated with MAP2 and FLAG (1:100 primary, 1:200 anti-mouse Cy3 secondary) primary antibody solution for one hour, and then incubated with secondary for 45 minutes in the dark. For actin/tubulin staining, cells were incubated in rhodamine-conjugated phalloidin (1:25) (a kind gift provided by Dr. Shakes, Life Technologies) for 1.5 hours in the dark, and then incubated in β-tubulin-FITC (1:150) for 1 hour in the dark. A detailed protocol of the staining procedure is included in the appendix.
RESULTS

MK-STYX induces neuronal differentiation

Previous work conducted by Flowers et. al showed that MK-STYX is expressed endogenously in PC12 cells, suggesting it could play a natural role in regulating signal transduction cascades (citation). Pilot studies suggested that MK-STYX alone induced cells to develop neurite outgrowths. Based on this observation, we wanted to observe if there was a synergistic effect between MK-STYX and nerve growth factor stimulated cells, resulting in an increase in neurite outgrowth.

PC12 cells were treated with 100 ng/ml of NGF to determine if MK-STYX enhanced the effect of NGF stimulation. We found that cells expressing MK-STYX were more likely to have neurites than the control, suggesting that MK-STYX sustains MAPK activity. In addition, cells expressing MK-STYX\textsubscript{active}, the catalytically active MK-STYX mutant, had less neurites than the control both in the presence and absence of NGF. (Figure 5)

MK-STYX alters primary neurite distribution

A common trend that we noticed during our experiments was that PC12 cells expressing MK-STYX were more likely to have multiple primary neurites originating from the cell body. This suggests that MK-STYX may have some effect on the ‘patterning’ of the neurite outgrowths. As mentioned previously, proper growth of dendrites is important in establishing and maintaining healthy neurons, and defects in dendritic growth can be associated with certain types of mental retardation, such as Down’s syndrome (Fiala et al., 2002). Thus, we wanted to quantify whether or not MK-STYX shifted the distribution of primary neurites.

PC12 cells were transfected with MK-STYX or pMT2, then either NGF stimulated or
not, and allowed to grow for 72 hours. In order to quantify whether MK-STYX changed the neurite outgrowth patterning of PC12 cells when they differentiated, only those cells bearing at least one neurite 20 µm in length were scored. Cells were then scored for the number of primary processes meeting the length criterion they possessed. In the absence of NGF, MK-STYX caused a significant shift in the distribution of cells containing one, two, or three neurites compared to the control (Figure 6). In the presence of NGF, MK-STYX also caused a significant shift in the distribution of cells containing one, two, or three neurites (Figure 7). Within the most extreme end of the spectrum (4+ neurites), there were no significant changes in these groups. This could be due to physiologic constraints, such as competition for space with other neurites. This suggests that MK-STYX changes the pattern of the neurites we see when PC12 cells differentiate, in addition to inducing initial neurite formation.

**MK-STYX alters neurite branching**

Given that MK-STYX shifted the distribution of primary neurites both in the absence and presence of NGF, we wanted to examine whether MK-STYX also affected the patterning of these cells when they differentiate by affecting branching. PC12 cells were transfected with MK-STYX or pMT2, then either NGF stimulated or not, and allowed to differentiate for 72 hours. In order to quantify whether MK-STYX changed the propensity of differentiated PC12 cells to have branching processes, cells were scored at the same time as primary neurites. This ensured that for each cell scored, we would have counted both its primary neurites and the number of branches. Only the processes emerging from primary neurites at least 20µm in length were classified as a branching neurite. In the absence of NGF, MK-STYX caused a significant decrease in the number of cells lacking any branch point compared to the control, suggesting that PC12 cells overexpressing MK-STYX do have a higher propensity to branch than control cells in the
absence of NGF stimulation (Figure 8). In the presence of NGF, MK-STYX caused a significant decrease in the number of cells lacking any branch point compared to the control. However, stimulation with NGF caused cells overexpressing MK-STYX to have a significant increase in the number of cells with one or four branches. Taken together with the effect of MK-STYX on primary neurite distribution, it is possible that this increase in branching is suggestive that these cells may be capable of forming functional connections with one another.

**MK-STYX affects the cytoskeleton in developing neurites**

In order to determine whether MK-STYX affected actin dynamics in differentiating PC12 cells, we decided to look at the distribution of tubulin and actin. PC12 cells were transfected with MK-STYX or pMT2, then either NGF stimulated or not, and allowed to differentiate for 72 hours. Cells were then fixed in formaldehyde and immunostained with rhodamine-conjugated phalloidin to label actin, and anti-β-tubulin conjugated to FITC to label microtubules. In the absence of NGF, PC12 cells overexpressing MK-STYX showed a more intense labeling of tubulin than actin (Figure 9). In addition, actin staining was confined primarily to the cell body, and very little in the neurites. Conversely, PC12 cells expressing the pMT2 control vector stained more intensely for actin than tubulin. In addition, control cells appeared to have actin ‘ruffling’, which indicates inefficient lamellipodia adhesion and inhibited actin filament turnover (Borm et al., 2005). This ruffling is expected in the control, as inhibition of actin filament turnover prevents initiation of neurite outgrowth. Upon stimulation with NGF, cells overexpressing MK-STYX had a much more robust staining for actin and tubulin, and actin was present throughout the length of the neurite. Cells expressing the pMT2 control vector had staining for both tubulin and actin; however, actin was primarily stained in the cell body and the beginning portions of
neurites (Figure 9). In terms of actin/tubulin distribution, neurites induced by MK-STYX overexpression independent of NGF stimulation resemble those of neurites induced by NGF stimulation in the control cells.

Something that we noticed in these experiments was that in the presence of NGF, cells overexpressing MK-STYX had areas of actin rich protrusions along the neurite. In order to get a better view of the actin distribution in these areas, we took images from the distal ends of neurites, as well as along the neurite shaft. In the distal ends of developing neurites, cells overexpressing MK-STYX and stimulated with NGF had a veil-like actin protrusion (Figure 10). Meanwhile, cells expressing the pMT2 control vector did not have these actin rich areas in the ends of their neurites. This type of actin protrusion is indicative of growth cones in neurites. MK-STYX induced the formation of more growth cones than the control, perhaps providing a causative explanation for why we see more primary neurites in cells expressing MK-STYX than the control. In addition, these growth cones are still ‘active’ after 72 hours, suggesting that these neurites are still elongating.

While the presence of more active growth cones in PC12 cells overexpressing MK-STYX may provide an explanation for why we see primary neurites, they do not necessarily explain why we see more branching. We then decided to look along the length of the neurite, where we saw additional actin protrusions. While staining was not intense along the length of the neurite, in cells over-expressing MK-STYX and stimulated with NGF, we do see small protrusions of actin (Figure 11). These are not present in cells expressing the control vector. We surmised that these protrusions could be dendritic spines or the site of branching. As mentioned earlier, dendritic branching is the result of filopodia stabilization early in neuronal development, while dendritic spines are the result of filopodia stabilization late in neuronal development. Given that
dendritic branching occurs via protrusion of actin along the neurite shaft, rather than by growth cone splitting in axons, it is possible that these actin protrusions could be the formation of new branches (Scott & Luo, 2001).

**MK-STYX affects MAP2 and Tau localization**

In order to assess the distinction between axon or dendrite-like processes in PC12 cells overexpressing MK-STYX or pMT2 control vector, we stained and determined the localization of MAP2 and Tau in neurite processes. MAP2 is used as a marker for dendritic processes, and thus serves as a postsynaptic marker, while Tau is a marker for axonal processes and serves as a presynaptic marker. PC12 cells were transfected with MK-STYX or pMT2 control vector, stimulated or not with NGF, and allowed to differentiate for 72 hours. Cells were then fixed and stained with MAP2 primary/α-rabbit Cy5 secondary and Tau primary/α-mouse Cy3 secondary antibodies. Slides were then visualized via fluorescent microscopy to determine the localization of MAP2 and Tau.

In all conditions, MAP2 and Tau were localized to the cell body and all major processes, with the exception of pMT2 transfected cells without NGF stimulation (Figure 12). In the non-stimulated pMT2 control, both MAP2 and Tau immunoreactivity were decreased compared to all other conditions. In non-stimulated cells overexpressing MK-STYX, Tau immunoreactivity was higher in the distal ends of some neurites, potentially distinguishing these processes as axonal. In NGF cells overexpressing MK-STYX, processes with a higher Tau immunoreactivity connected to processes with a higher MAP2 immunoreactivity. In addition, these cells formed a branching network in which an asymmetric distribution of Tau and MAP2 contributes to the connection of a MAP2 positive neurite with a Tau positive neurite. In the NGF stimulated pMT2 controlled, MAP2 and Tau immunoreactivity had a more symmetric distribution. As such, these processes
were less likely to form contacts with a neurite harboring a stronger immunoreactivity for the opposite protein (i.e., MAP2 neurites seldomly connected with Tau neurites, and vice-versa).

**MK-STYX colocalizes with MAP2**

So far, it seems that MK-STYX induced neurites in both the presence and absence of NGF have properties that are characteristic of dendritic processes. Namely, a higher number of primary neurites and branching, actin rich processes along the neurite shaft, and an asymmetric distribution of MAP2 and Tau. We asked what the localization of MK-STYX would be in these neurite processes, and more specifically, does MK-STYX co-localize with MAP2? To answer this question, PC12 cells were transfected with MK-STYX or pMT2 control vector, stimulated or not with NGF, and allowed to differentiate for 72 hours. Cells were then fixed and stained with MAP2 primary/α-rabbit Cy5 secondary and FLAG primary/α-mouse Cy3 secondary antibodies. FLAG was used to detect overexpressed MK-STYX. Since the pMT2 control vector lacks a FLAG tag, immunoreactivity should be largely absent asides from non-specific staining.

In both the presence and absence of NGF, MAP2 localization was similar as in the MAP2/Tau localization experiments conducted previously. Namely, MAP2 was evenly distributed throughout the cell body and neurite processes. In the absence of NGF, however, FLAG immunoreactivity is not consistent throughout the whole length of a neurite process, with a majority of MK-STYX being located in the cell body and the beginning portions of a neurite (Figure 13). As such, MAP2 and MK-STYX colocalization are limited to these areas. In the presence of NGF, however, MK-STYX is distributed along the length of the neurite, as well as in minor processes. In these neurites, there is a strong colocalization of MAP2 and MK-STYX. It is possible that there is a synergistic effect between MK-STYX and NGF stimulation, both serving to augment the other. Since MK-STYX colocalizes with MAP2 in the minor processes and
branches upon NGF stimulation, this validates the previous data suggesting that MK-STYX induces more branches in the presence of NGF.

**MK-STYX regulates cofilin activity via phosphorylation**

Since it was previously shown that MK-STYX decreases RhoA activation (Flowers et al., 2014), we wanted to know whether MK-STYX had an effect on downstream effectors of RhoA. We chose cofilin, an actin binding and severing protein whose activity is mediated by RhoA. In its non-phosphorylated state, cofilin is ‘active’, and has the capability to sever actin, allowing for it to mediate cytoskeletal remodeling. When phosphorylated by LIMK, cofilin is considered inactive, allowing for filament stability, and persistence of neurites. Since LIMK is activated by ROCK, which in turn is activated by RhoA, we proposed that MK-STYX would affect cofilin phosphorylation. To analyze the phosphorylation patterns of cofilin, cells were transfected with MK-STYX, scrambled control, or MK-STYX shRNA-A, and stimulated with 100 ng/ml NGF for 24 hours or not. In the absence of NGF stimulation, cofilin phosphorylation was decreased in cells over-expressing MK-STYX or scrambled control (Figure 14). Meanwhile in cells where MK-STYX was knocked down via shRNA, cofilin phosphorylation increased. This supports previous work by Flowers et al. which showed knockdown of MK-STYX prevents NGF stimulated PC12 neurite extensions (citation). Since cofilin is phosphorylated from the beginning, it is possible that there is no initial cytoskeletal reorganization required to induce neurite formation. 24 hours post NGF stimulation, cofilin phosphorylation in cells over-expressing MK-STYX was higher than in the scrambled control and MK-STYX shRNA-A. A subsequent deactivation of cofilin via phosphorylation allows filament stability and prevents neurite retraction, providing a scaffold for continual actin and microtubule polymerization.
Given that MK-STYX did have an effect on the phosphorylation status of cofilin depending upon NGF stimulation, we wondered what the changes in cofilin phosphorylation would be over time. We hypothesized that following a brief period of activity, cofilin would be phosphorylated earlier than the control, providing a ‘scaffold’ for continual neurite outgrowth. However, given that MK-STYX also induces neurite branching in PC12 cells, there must be a dephosphorylation at some point in order to mediate actin nucleation and branching. To analyze the phosphorylation patterns of cofilin over time, cells were transfected with MK-STYX or pMT2 control vector. Post-transfection, cells were stimulated with 100 ng/ml NGF for 0, 3, 5, 12, or 30 minutes, and 24, 48, or 72 hours, and then lysed for immunoblotting. In cells over-expressing MK-STYX, cofilin phosphorylation was initiated 3 minutes post NGF stimulation, reaching noticeable levels at 30 minutes (Figure 15). In cells transfected with pMT2 control vector phosphorylation was not noticeable until 24 hours post NGF stimulation. Intriguingly enough, in cells overexpressing MK-STYX, cofilin dephosphorylation was noticed at 72 hours, while in the control, cofilin phosphorylation was still increasing. Cofilin dephosphorylation is mediated by chronophin and the slingshot family of phosphatases. Immunoblotting showed that chronophin expression was relatively stable over the course of NGF stimulation. However, MK-STYX may affect the activity of the slingshot phosphatases or LIM kinase, thus mediating cofilin phosphorylation and dephosphorylation. Taken together, these data suggest that MK-STYX may play an important role in cofilin activation and inactivation, playing an important role in actin filament dynamics.
DISCUSSION

PC12 cell differentiation

The PC12 cell line is a model system used widely in the study of neuronal differentiation. The neurotrophin NGF binds to the Trk receptor, inducing sustained activation of ERK1/2. Sustained activation of ERK1/2 causes a cessation of cellular proliferation and the induction of neurite outgrowths. Here, we have shown that MK-STYX plays a role in the induction of PC12 neuronal differentiation. Prior studies have confirmed that MK-STYX is endogenously expressed in PC12 cells, and here we have shown again that overexpression of MK-STYX induces neuronal outgrowths in PC12 cells (Flowers et al., 2014). Prior studies also have shown that MK-STYX enhances the effects of NGF by promoting longer neurite outgrowths in cells stimulated with NGF. This suggests that there may be a synergistic effect between MK-STYX and NGF, both augmenting the other to produce a more robust differentiation phenotype.

We have previously shown that MK-STYX induces neurite outgrowths through inactivation of RhoA (Flowers et al., 2014). RhoA inactivation is required for the induction of neurite outgrowths, whereas activation of RhoA promotes neurite outgrowth extension (Sebok et al., 1991). This temporal regulation of RhoA activity is intriguing, as it could be responsible for the formation of neurite branches. As such, we have been able to use these studies to pursue other avenues of research, such as whether MK-STYX induced neurites have a distinct branching pattern.

Neurite branching

Dendrites differ from axons in many respects, both morphologically and functionally. In addition, dendrites have specialized structures such as spines and interstitial branches that are not
found in axons. So, how do neurons acquire their dendritic morphology? What controls the extent to which branching may occur? While most studies of dendrite branching have used *Drosophila* as a model organism, several key players are important in regulating neuronal morphology. Transcription factors, signaling pathways, cytoskeletal elements, and even endosomes have been identified as contributors to dendrite patterning (Jan and Jan, 2010).

In order to function properly, neurites must follow several physiological requirements. First, dendrites need to cover an area that encompasses its synaptic inputs (Wassle and Boycott, 1991). This means that dendrites must be able to form viable connections with axons. Second, the dendrites must branch sufficiently in order to receive signals that converge onto it. This means that synaptic contacts must be established from the meeting of the presynaptic regions of axons and the postsynaptic region of dendrites in order to correctly process signals. Finally, dendrites must have some flexibility to adjust during their development. In this case, the cytoskeleton must remain dynamic. We believe that neurites induced by MK-STYX meet these physiological requirements. The first requirement, area covering, is met by previous studies that showed that MK-STYX induced longer neurite outgrowths in PC12 cells than the control (Flowers et al., 2014). This allows these neurites to cover a greater area, thus increasing the likelihood that they will meet with the axon of another cell and form viable connections. The second requirement, sufficient branching, is shown by the primary and secondary neurite data presented in this thesis. Given that cells overexpressing MK-STYX have more primary neurites as well as branches, there is a higher likelihood of synaptic contacts being established upon the union of a pre and postsynaptic region. Put simply, overexpressing MK-STYX allows these cells more chances to have viable connections. The third requirement, a dynamic cytoskeleton, is also displayed in PC12 cells overexpressing MK-STYX. In the presence of NGF, we see more growth
cones as well as actin protrusions along the neurite shaft that are characteristic of dendritic spines or future branches. Cells expressing MK-STYX, therefore, still have enough plasticity to respond to the neuronal environment, and reorganize the dendritic arbor as necessary. Given that PC12 cells overexpressing MK-STYX meet the physiological requirements to ensure proper neuronal function, it is prudent to further characterize the role of MK-STYX in the establishment of synaptic contacts.

**Signaling to the actin cytoskeleton**

The actin cytoskeleton is the key to every step in the changes of neurons during development. Key regulators of the actin cytoskeleton are the Rho GTPases, and these pathways play important roles in neuronal morphogenesis. Thus, this section will connect previous results showing that MK-STYX decreases RhoA activation (Flowers et al., 2014) with the current work highlighting the effects of MK-STYX in neuronal morphology and cofilin signaling.

When bound to GTP, Rho GTPases are capable of activating downstream effectors that regulate the actin cytoskeleton. Cdc42, a member of the Rho GTPases, plays a role in de novo actin polymerization through the Arp2/3 complex (Luo, 2002). Arp2/3 is an actin binding protein that has an affinity to bind to the sides of actin chains. However, Arp2/3 alone is intrinsically inactive, and is unable to promote actin nucleation on its own. However, WASP (Wiskott-Aldrich syndrome protein), can bring actin monomers and Arp2/3 together, thus activating Arp2/3 and allowing for branching. WASP is usually locked in a confirmation that results in auto-inhibition. However, Cdc42 binding releases it from this auto-inhibitory conformation, allowing WASP to stimulate Arp2/3 and allow for de novo actin polymerization (Rohatgi et al., 1999). Perhaps MK-STYX induced branching could be a result of increased actin nucleation mediated by Arp2/3/WASP?
One of the most exciting findings in our current research is the mediation of cofilin phosphorylation/dephosphorylation in cells overexpressing MK-STYX, supporting the potential for MK-STYX to modulate spatial/temporal regulation of cofilin phosphorylation in PC12 cells. Cofilin/ADF (actin depolymerization factor) is one of the most characterized proteins that mediate actin depolymerization. Cofilin is capable of depolymerizing F-actin at the minus end and is also capable of actin severing. In the case of severing, ADP-actin filaments are broken off, which can then be used for actin branching and nucleation by the Arp2/3 complex. Cofilin activity is regulated by phosphorylation at serine 3, phosphorylation of this residue inhibits cofilin activity and thus prevents actin severing (Bamburg, 1999).

We found that in cells over-expressing MK-STYX, cofilin phosphorylation was initiated 3 minutes post NGF stimulation, reaching noticeable levels at 30 minutes. Curiously enough, in cells overexpressing MK-STYX, cofilin reverted back to its active, dephosphorylated form at 72 hours, while in the control, cofilin phosphorylation was still increasing. What could be causing this spatial/temporal regulation of cofilin phosphorylation? As it turns out, both LIM kinase (LIMK) and Slingshot (SSH) are both necessary in order for neurite formation (Endo et al., 2007). This supports a model in which both cofilin phosphorylation and dephosphorylation are required in order to regulate neurite formation. SSH is a specific cofilin phosphatase that is capable of dephosphorylating cofilin. To date, three SSH isoforms have been implicated in cofilin dephosphorylation: hSSH1, hSSH2, and hSSH3, all of which have been implicated in neurite extension (Endo et al., 2007). LIMK is a kinase that is capably of phosphorylating cofilin at serine 3. LIMK can be phosphorylated by the Pak kinases, as well as ROCK, which increase its kinase activity and phosphorylation of cofilin (Edwards et al., 1999) Pak, a downstream effector of Rac and Cdc42, can activate LIM-kinase, leading to down regulation of cofilin
severing and actin depolymerization. LIMK is the unifying factor between the Rho, Rac, and Cdc42 GTPases, at this point they can all cause actin stabilization by inactivation of cofilin via LIMK phosphorylation. Given that both LIMK and SSH are necessary to induce neurite formation, it could be possible that MK-STYX is regulating cofilin phosphorylation via one of these two proteins, or possibly both. So, what is the functional relevance of cofilin in neurons? It turns out that unphosphorylated cofilin expression is increased in the growth cones of primary hippocampal neurons, as well as cofilin overexpression can increase neurite outgrowth (Bamburg & Bray, 1987; Meberg & Bamburg, 2000). Since we have shown MK-STYX has a differential effect on cofilin phosphorylation compared to control cells, this may be the reason we see more active growth cones in NGF stimulated cells expression MK-STYX, as well as longer neurites.

Summary and Future Directions

This study has further characterized the role of MK-STYX in neuronal differentiation. This data provides evidence that over-expression of MK-STYX can cause neuronal differentiation, MK-STYX affects the distribution of primary and secondary neurite, MK-STYX affects actin dynamics, MK-STYX-induced neurites have pre and postsynaptic qualities, and that MK-STYX regulates cofilin phosphorylation. These studies implicate a potential therapeutic use of MK-STYX. Disruption of proper protein phosphorylation and dephosphorylation leads to neurological diseases such as Alzheimer’s. Additionally, proteins associated with presynaptic vesicles, such as Tau, have been shown to be hyperphosphorylated in those diagnosed with Alzheimer’s (Hu et al., 2002). Gaining further insight into the role of MK-STYX in neuronal differentiation may lead to further understanding of neurological disorders. Further studies of MK-STYX in PC12 neuronal differentiation will focus on understanding the mechanisms of RhoA inactivation, cofilin phosphorylation, and further characterization of its neuronal effects.
Future Aim 1: While we know that MK-STYX decreases RhoA activation, we do not know how this decrease in activity is achieved. Is it possible that MK-STYX is directly interacting with RhoA, serving to block its function? Or is it working upstream through this GTPase’s respective GAP or GEF? It may also be possible that MK-STYX is not directly blocking this pathway, rather, this decrease in RhoA activity may be the result of a direct interaction with RhoA causing differential localization of RhoA in cells overexpressing MK-STYX. To determine the method that MK-STYX decreases RhoA activation, we will perform pull-down assays to determine whether MK-STYX is directly interacting with RhoA, and then co-localization studies to determine whether or not MK-STYX causes a differential localization of RhoA in PC12 cells.

Future Aim 2: A final avenue of research is to determine how MK-STYX affects actin activity via cofilin dynamics. Cofilin, a member of a larger group of proteins characterized as actin binding proteins, can act in concert with other regulatory proteins to mediate the response of the actin cytoskeleton to extracellular signals. By binding the barbed end of actin, cofilin can have actin severing activity or nucleation activity depending on its phosphorylation status. In order to monitor the effect of MK-STYX on the actin binding and severing activity of cofilin over time, F-actin/cofilin spin down assays followed by SDS-PAGE analysis can be performed. In addition, determining the effects of MK-STYX on LIMK and SSH will be crucial in determining how MK-STYX temporally regulates cofilin phosphorylation.
Figure 5. MK-STX induces neurite outgrowths in the absence and presence of NGF.

PC12 cells were transfected with pEGFP and pMT2, pEGFP and MK-STYX, or pEGFP and MK-STYX\textsubscript{active}. 24 hours post-transfection, PC12 cells were either not stimulated or stimulated with 100ng/ml of NGF. (A) 72 hours post-transfection or 72 hours post stimulation for the NGF stimulated group, cells were scored for the presence of neurite extensions greater than 20 \( \mu \text{m} \) in length. Statistical analysis was performed using two-way ANOVA (No stimulation: p<0.001; NGF stimulation: p<0.0001) (B) Live PC12 cells were imaged using phase and fluorescence microscopy to illustrate the growth of neurites in PC12 cells following transfection and subsequent NGF stimulation. PC12 cells expressing MK-STYX appeared to have more neurites than the control. Three replicates of this experiment were performed, n=100.
Figure 6. MK-STYX shifts the distribution of primary neurites in the absence of NGF.

(A). Differentiated PC12 cells expressing MK-STYX or pMT2 co-transfected with pEGFP were scored to determine the primary neurite distribution 72 hours post-transfection. MK-STYX caused a significant shift in the distribution of one, two, and three neurites when compared to the control (1 neurite: p=0.0013, 2 neurites: p=0.0056, 3 neurites: p=0.0097). Statistical analysis was done by t-test (Sidak’s multiple comparison) between the two groups. (B). Live PC12 cells were imaged using phase and fluorescence microscopy to illustrate the growth of neurites in PC12 cells that were transfected and allowed to differentiate for 72 hours. Four replicates of this experiment were performed, n =100.
Figure 7. MK-STYX shifts the distribution of primary neurites in the presence of NGF.

(A) Differentiated PC12 cells expressing MK-STYX or pMT2 co-transfected with pEGFP were scored (n = 100) to determine the primary neurite distribution after stimulation with 100 ng/ml NGF for 72 hours. MK-STYX caused a significant shift in the distribution of one, two, and three neurites when compared to the control (1 neurite: \( p = 0.0039 \), 2 neurites: \( p = 0.002 \), 3 neurites: \( p = 0.0005 \)). Statistical analysis was done by t-test (Sidak’s multiple comparison) between the two groups. Four replicates of this experiment were performed. (B) Live PC12 cells were imaged using phase contrast and fluorescence microscopy to illustrate the growth of neurites in PC12 cells that were transfected and allowed to differentiate for 72 hours. Arrowheads indicate which neurites were scored as primary (originating from the soma) neurites. In this example, there are four primary neurites. Neurites with bifurcations close to the soma were counted as one neurite for these purposes. Four replicates of this experiment were performed.
Differentiated PC12 cells expressing MK-STYX or pMT2 co-transfected with pEGFP were scored to determine the secondary branch distribution in non-stimulated (A) and NGF stimulated (B) PC12 cells. In the absence of NGF, MK-STYX only induced a significant decrease in the number of cells lacking any bifurcations when compared to the control (0 branches: p=0.04). In the presence of NGF, MK-STYX induced a significant decrease in the number of cells lacking any bifurcations when compared to the control (0 branches: p=0.004), while significantly increasing the number of cells with 1, or 4 branches (1 branch: p=0.04, 4 branches: p=0.01). While there was a noticeable increase in the number of cells with 2 or 3 branches when MK-STYX is overexpressed, these groups are not statistically significant (2 branches: p=0.06, 3 branches: p=0.08). Statistical analyses were performed via t-tests between the control and experimental groups in both conditions. Four replicates of this experiments were performed, n = 100.
**Figure 9. MK-STYX affects tubulin and actin dynamics in PC12 cells.**

PC12 cells were transfected with MK-STYX or pMT2 control vector and stimulated with 100 ng/ml of NGF for 72 hours or not. Following NGF stimulation, cells were fixed and sequentially stained with rhodamine conjugated phalloidin (actin), anti-β-tubulin conjugated FITC antibody, and DAPI. Slides were analyzed by fluorescence microscopy. Merged images show the distribution of tubulin and actin relative to each other. Phase image shows the cell morphology. Experiments were repeated three times.
Figure 10. PC12 cells overexpressing MK-STYX have active growth cones in the distal end of developing neurites.

PC12 cells were transfected with MK-STYX or pMT2 control vector and stimulated with 100 ng/ml of NGF for 72 hours or not. Following NGF stimulation, cells were fixed and sequentially stained with rhodamine conjugated phalloidin (actin), FITC-conjugated β-tubulin antibody, and DAPI. Slides were analyzed by fluorescence microscopy. To visualize actin distribution in developing neurites, images were taken from the distal ends of elongating neurites. Actin rich protrusions at the tip of a developing neurite are suggestive of an active growth cone. Merged images show the distribution of tubulin and actin relative to each other. Phase image shows the cell morphology.
**Figure 11.** PC12 cells overexpressing MK-STYX have actin-rich protrusions along the neurite shaft.

PC12 cells were transfected with MK-STYX or pMT2 control vector and stimulated with 100 ng/ml of NGF for 72 hours or not. Following NGF stimulation, cells were fixed and sequentially stained with rhodamine conjugated phalloidin (actin), FITC-conjugated β-tubulin antibody, and DAPI. Slides were analyzed by fluorescence microscopy. To visualize actin distribution in developing processes resembling dendrites, images were taken along the neurite shaft. Actin rich protrusions along the length of a neurite shaft indicate interstitial branching, which is characteristic of dendritic arborization and branching. Merged images show the distribution of tubulin and actin relative to each other. Phase image shows the cell morphology.
Figure 12. MK-STYX affects MAP2 and Tau localization in PC12 cells.

PC12 cells were transfected with MK-STYX or pMT2 control vector and stimulated with 100 ng/ml of NGF for 72 hours or not. Following NGF stimulation, cells were fixed and stained for the dendritic and axonal markers, MAP2 and Tau, respectively. MAP2 immunoreactivity (green) was localized in the cell body and major processes, while Tau immunoreactivity (red) is stronger in presynaptic terminals. In non-stimulated cells overexpressing MK-STYX, Tau immunoreactivity is higher in the distal end of neurites. In non-stimulated cells expressing pMT2 control vector, tau immunoreactivity is greatly decreased. In NGF stimulated cells overexpressing MK-STYX, processes with higher Tau immunoreactivity were connected to processes with higher MAP2 immunoreactivity. In the control NGF stimulated cells, immunoreactivity was more evenly distributed along the length of the neurite. Experiments were repeated five times. Phase image shows the cell morphology.
Figure 13. MK-STYX co-localizes with MAP2.

In order to determine whether MK-STYX co-localized with the dendritic marker MAP2, PC12 cells were transfected with MK-STYX or pMT2 control vector and stimulated with 100ng/ml of NGF for 72 hours or not. Cells were fixed and stained for MAP2 and FLAG to detect overexpressed MK-STYX. In non-stimulated cells overexpressing MK-STYX, MAP2/MK-STYX co-localization was confined primarily to the cell body and the portion of the neurite closest to the cell body. Upon NGF stimulation, MAP2 and MK-STYX strongly colocalize along
the entire length of the neurite. Experiments were repeated three times.

**Figure 14. MK-STYX affects cofilin phosphorylation.**

PC12 cells were transfected with MK-STYX, scrambled shRNA, or MK-STYX shRNA, stimulated with NGF or not, and lysed 24 hours afterwards for immunoblotting. Anti-phospho cofilin antibody showed that MK-STYX decreased cofilin phosphorylation in non-stimulated cells compared to MK-STYX shRNA-A. Upon stimulation with NGF, cofilin phosphorylation increased relative to the MK-STYX shRNA-A, which did not have a noticeable increase in phosphorylation. Blots were stripped and probed for cofilin and β-tubulin as a loading control. STYXL1 antibody showed over-expressed MK-STYX relative to the scrambled control, while endogenous MK-STYX was down-regulated by MK-STYX shRNA-A compared to the scrambled control. The blot was stripped and probed with anti-FLAG to detect over-expressed MK-STYX. Three replicates of this experiment were performed. Published in Flowers, Rusnak, Wong, Banks, et al., PLOS ONE, 2014.
Figure 15. MK-STYX affects cofilin phosphorylation over the course of NGF stimulation.

PC12 cells were transfected with pMT2 or MK-STYX control vector. 24 hr post-transfection cells were stimulated with 100 ng/ml NGF or not for the indicated time points, lysed, and immunoblotted. MK-STYX started to increase cofilin phosphorylation in NGF stimulated cells beginning at 3 minutes, peaking at 48 hours, and then decreasing by 72 hours. In contrast, cofilin phosphorylation did not increase until 24 hours of NGF stimulation, and continued to increase. These blots were stripped and probed for cofilin as a loading control. Chronophin, a cofilin phosphatase, had expression levels suggesting that the decrease in cofilin phosphorylation at 72 hours in cells expressing MK-STYX is independent of cofilin phosphatase activity. There appears to be a decrease in chronophin phosphatase activity at 48 and 72 hours in cells expressing MK-STYX. The blot was stripped and probed with anti-FLAG to detect over-expressed MK-STYX, and probed for anti-β tubulin as a loading control. Three replicate experiments were performed.
APPENDIX

Buffer Preparation for Immunostaining PC12 cells

Background: A problem with the PC12 cell line is that, unlike HeLas, they do not strongly adhere to their substrate. This is particularly troublesome when trying to fix cells, as they slide off glass coverslips. Coating coverslips with collagen can help promote adhesion. However, it alone is not enough to retain a large amount of viable PC12 cells. Thus, we use a phosphate buffered saline solution containing Ca^{2+} and Mg^{2+} ions. Ca^{2+} and Mg^{2+} ions are required for cell-cell and cell-substrate adhesion. Supplementing D-PBS with Ca^{2+} and Mg^{2+} ions helps to maintain the structural integrity of cells better than D-PBS alone by strengthening the connections between the extracellular matrix and integrins. When immunostaining, use this solution for the fixation (if using formaldehyde), permeabilization, blocking, antibody dilution, and washing solutions. Ca^{2+} and Mg^{2+} also help to keep chromatin intact and within the nucleus during the staining procedure, so it helps to reduce background fluorescence and fluorescence bleed-through when using DAPI. Since these ions inhibit cell-cell and cell-substrate detachment, it is not suggested to use this process for trypsinization; however, it is useful for live cell imaging for short periods of time.

Preparation of 10X D-PBS – Bring to 1 Liter with ddH_{2}O
Reagents:
- 2 grams KCl (Final concentration: [27 mM])
- 2 grams KH_{2}PO_{4} (Final concentration: [14.7 mM])
- 80 grams NaCl (Final concentration: [1370 mM])
- 21.6 grams Na_{2}HPO_{4}-7H_{2}O (Final concentration: [162 mM])

Preparation of 10X MgCl_{2} – Bring to 100 ml with ddH_{2}O
Reagents:
- 1 gram MgCl_{2}-6H_{2}O (Final concentration: [4.92 mM])

Preparation of 10X CaCl_{2} – Bring to 100 ml with ddH_{2}O
Reagents:
- 1 gram CaCl_{2} (Final concentration: [9.01 mM])

Note: Do not take shortcuts when preparing D-PBS containing CaCl_{2} and MgCl_{2}. Do not autoclave the Calcium solution and the Magnesium solution together, as this will result in an unfilterable precipitate. All solutions must be made separately!

Preparation of 1X D-PBS containing 100mg/L CaCl_{2} and MgCl_{2}:
- Take 1 volume (100ml) of 10X PBS; bring to 800 ml with dI.
- Take 1 volume (10ml) of 10X CaCl_{2}; bring to 100 ml with dI
- Take 1 volume (10ml) of 10X MgCl_{2}; bring to 100 ml with dI.
N.B. – If this solution needs to be sterile, you may autoclave each of the 1X components separately at this point. I find it easier to make 200 ml of the calcium and magnesium solutions, that way you have some ready for the sterile technique (next page).

If solution needs to be sterile, under the TC hood:
   - Add 100 ml of MgCl₂ solution to 1X D-PBS. Pour slowly with swirling to avoid precipitation of magnesium.
   - Then add 100 ml of CaCl₂ solution to the 1X D-PBS containing magnesium. Take caution to add slowly, if precipitate starts to form, stop, wait for it to dissolve, and then continue adding.
N.B. – Never add the CaCl₂ solution first as it may react with the phosphate in the D-PBS and form a precipitate of calcium phosphate.

If solution do not need to be sterile (i.e., no TC use): Follow steps as above at lab bench.

**PC12 immunostaining protocol**

**Important:** Prepare all reagents for immunostaining and fixation in 1X D-PBS with 100 mg/L MgCl₂ and CaCl₂!

Notes about prefixation: When withdrawing all the culture medium from a well, the rapid change in surface tension, as well as loss of Ca²⁺ and Mg²⁺ ions causes cells to lose their shape and dissociate from their substrate. By prefixing, we are slightly fixing the cells so they are much more ‘rigid’. In the case of formaldehyde and methanol, proteins start to become cross-linked and are much more rigid. In the case of acetone, proteins precipitate. Both of these help to contribute to cell stability upon removing culture medium, and also helps them to stay better adhered to their substrate.

**If fixing with acetone:**

- Aliquot 100% acetone, store at -20°C until ready for use.

- Prefix the cells by adding ½ volume of acetone to plates containing culture medium (i.e., if using 3 ml of medium, add 1.5 ml of pure acetone to each well). Prefix for 2 minutes at -20°C. Acetone causes fixation by precipitating proteins, so you will see precipitate due to the acetone also acting upon the proteins in the culture media. This is normal, and will be washed away upon aspiration and washes.

- Withdraw acetone/culture medium mixture, and add 1 ml of cold acetone to each well. Fix at -20°C for 8 minutes

- At this point proceed with immunostaining protocol. Acetone also acts as a permeabilization reagent, so please skip the permeabilization step.
-N.B.: I do not recommend using acetone to fix if you are fixing slides that are co-transfected with a fluorescent protein. The precipitation caused by acetone seems to reduce the amount of visible fluorescence we can obtain. In this case, it is best to use formaldehyde fixation.

*If fixing with formaldehyde:*

- Prepare two formaldehyde solutions: one is a 4% formaldehyde solution, and the other is a 3.7% formaldehyde solution.

- Leave culture medium in wells. Add an equivalent amount of 4% formaldehyde solution to each well (i.e., if there are 3 ml medium/well, then add 3 ml 4% formaldehyde solution). Thus, the working concentration of formaldehyde in these wells is 2%. This is sufficient for prefixation for 2 minutes.

- Remove prefixation solution. Add 3.7% formaldehyde solution to each well. Fix for 10 minutes. Since we prefixed, do not fix any longer than 10-12 minutes as the proteins will become too cross-linked due to the formation of methylene bridges, and antigen sites for antibodies may become masked. Trypsin solutions can be used for antigen retrieval, but this process does not make the cells happy. Moral of the story: Don’t over fix your cells!

- Proceed to immunostaining protocol.

*If fixing with methanol (only use this process if it works for the antibodies you are immunostaining with):*

- Aliquot 100% methanol, store at -20°C until ready for use.

- Similar to the acetone fixation, prefix the cells for 2 minutes at -20°C.

- Withdraw prefix mixture, add 2 ml 100% ice cold methanol to each well. Fix for 15 minutes at -20°C.

- Methanol semi-permeabilizes the membrane, so it is a gentle fixation process for the cells. However, further permeabilization with Triton-X-100 is not recommended as you can end up over permeabilizing and ruining structure integrity. Methanol fixation is therefore good if you are staining for proteins located near the cell membrane, as antibodies will have greater access here.

*Begin immunostaining:*

*N.B. –* When adding wash buffer to wells, add slowly and carefully. Do not pipette directly onto coverslip. Instead, plant pipette onto the bottom of the well and add wash buffer slowly.

- After fixation, wash slides 2X in PBS with 100mg/L CaCl₂ (Final concentration: [9.01 mM]) and 100mg/L MgCl₂-6H₂O (Final concentration: [4.92 mM]) for 5 minutes.
For all aspirations, attach a gel loading tip to the Pasteur pipette. This prevents wash buffers from being withdrawn too fast and causing cells to detach from the coverslips.

- If fixing with formaldehyde, permeabilize by immersion in 0.2% Triton-X-100 for 5 minutes. Wash 2X with PBS for 5 minutes each, aspirate as above.

- Prepare primary antibody solution. If doing costaining experiments, and the antibodies are raised in different hosts (i.e., one mouse, one rabbit), you can make one solution containing both primary antibodies. If you have plenty of antibody (and it requires a very low concentration for staining), I suggest making 400 µl of solution for 6 coverslips, as this seems to stain better, and also makes coverslips easier to pick up to transfer from the humidified chamber. Antibody solution should have 1.5% goat serum, all components diluted in PBS containing Ca and Mg.

- In a humidified chamber, pipet 60-65 µl (If you made 400 total) of antibody solution onto the parafilm for each coverslip. Invert coverslips onto antibody solution, close the humidified chamber and incubate for 1-1.5 hours (If you have the time, 1.5 hours usually provides a better signal in the end). If using rhodamine-conjugated phalloidin, stain for 1.5 hours in the dark.

- Remove coverslips from humidified chamber, place cell-side up in six well plates. Wash 2X for 5 minutes each in PBS containing Ca and Mg.

- Prepare secondary antibody solution. If costaining experiments, and the secondary antibodies are different hosts (one mouse, one rabbit), you can make one solution containing both secondary antibodies. Antibody solution should have 1.5% goat serum, all components diluted in PBS containing Ca and Mg. For this step, it is probably better to only prepare 200 µl total for 6 coverslips. This helps to avoid background staining caused by the secondary antibody.

- In a humidified chamber, incubate coverslips with the secondary antibody solution for 45 minutes to 1 hour in the dark.

- Transfer coverslips back to the 6 well plate, cell-side up. Wash 2X for 10 minutes each in PBS containing Ca and Mg. The longer wash steps here help to remove more of the extra secondary antibody, without the repeated stress from multiple washes.

- Mount using GelMount containing DAPI. Store at 4°C in the dark. You can visualize the slides about 2-3 hours after mounting if the GelMount has dried. If doing actin stains, it is best to visualize the slides within the week they are prepared.
REFERENCES


