The Pseudophosphatase MK-STYX Induces Neuronal Differentiation in PC12 Cells

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The Pseudophosphatase MK-STYX Induces 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 and Mary

by

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MK-STYX [MAPK (mitogen-activated protein kinase) phosphoserine/threonine/tyrosine binding protein] is a pseudophosphatase of the MAPK phosphatase family. Though structurally related to the MAPK dual specificity phosphatases, MK-STYX lacks both the critical nucleophilic cysteine and adjacent histidine residues in the active site signature motif (HCX₃R) required for catalysis. Thus, MK-STYX is catalytically inactive. Despite its lack of catalytic activity, MK-STYX maintains its ability to bind phosphorylated proteins but not dephosphorylate them. This thesis focuses on the role of MK-STYX in neuronal differentiation signal transduction cascades. The rat pheochromocytoma (PC12) cell line was used as a model system to study neuronal differentiation. Prior studies have shown that stimulation by neurotrophin nerve growth factor initiates sustained activation of a Ras-dependent MAPK phosphorylation cascade. Specifically, it is the sustained activation of extracellular signal-regulated kinase (ERK) 1/2 that leads to neuronal differentiation in PC12 cells. The results presented here confirm that MK-STYX causes neuronal differentiation in PC12 cells, suggesting a role in modulation of the MAPK pathway. Initially, MK-STYX modulation of the small G-protein Ras was investigated, because activation of Ras is known to lead to activation of the MAPK signal transduction cascade. This thesis shows that MK-STYX causes a very transient decrease in the activation of Ras. To further investigate the role of MK-STYX in the MAPK cascade, the kinase activity of MEK was inhibited. Without MEK activation of ERK 1/2, PC12 cells should not be able to differentiate. However, despite the presence of an inhibitor, MK-STYX continued to induce neuronal differentiation, suggesting MK-STYX acts independently of the MAPK pathway. This finding led to investigation of the small G-protein, RhoA. RhoA is involved in actin cytoskeleton remodeling. Prior studies have shown that activation of RhoA inhibits the initiation of neuronal outgrowths, whereas inactivation of RhoA promotes it. These studies provide evidence that MK-STYX decreases activation RhoA leading to the induction of neurite outgrowth. In summary, this thesis demonstrates that MK-STYX can induce PC12 neuronal differentiation through inactivation of RhoA and independently of the MAPK pathway. This strongly supports a model in which the pseudophosphatase MK-STYX has a critical role as a regulator in PC12 neuronal differentiation.
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INTRODUCTION

Phosphorylation Signaling Cascades

Signal transduction cascades are critical for controlling and coordinating cellular responses. Protein phosphorylation is an important post-translational modification of these transduction cascades. Phosphorylation modifications induce reversible protein conformational changes to regulate protein activity. In mammalian cells, the hydroxyl group of threonine, serine, or tyrosine amino acid residues can be reversibly phosphorylated by kinases and phosphatases to control protein-protein interactions. Kinases catalyze the transfer of a phosphoryl group (PO$_3^{2-}$) from adenosine triphosphate (ATP) to the hydroxyl group of the target protein, whereas phosphatases catalyze the removal of the phosphoryl group and regeneration of the hydroxyl group (Roskoski, 2012). Protein phosphorylation and de-phosphorylation allow kinases to control the amplitude of a signal response and phosphatases to control the rate and duration of a response (Hornberg et al., 2005). This control of cellular signal responses is critical to the regulation of neuronal development. Pseudoenzymes such as pseudophosphatases and pseudokinases add another layer of complexity to the control of these signal transduction cascades. These pseudoenzymes are catalytically inactive but maintain the ability to bind target substrates. Therefore, it is critical to understand the role these pseudoenzymes play in the regulation of signal transduction cascades. This thesis focuses on the role of pseudophosphatase MK-STYX in neuronal differentiation signal transduction cascades.
The Rat Pheochromocytoma (PC12) cell line

The rat pheochromocytoma (PC12) cell line is a model system for the study of neuronal signal transduction. PC12 cells respond reversibly to stimulation by extracellular neurotrophins such as nerve growth factor (NGF) causing a cessation of cellular proliferation and induction of neuronal differentiation into sympathetic-like neurons (Fujita et al., 1989). Unlike sympathetic neurons, PC12 cells do not require NGF for survival (Green and Tischler, 1976). NGF mediates a cellular response through binding receptor tyrosine kinases (RTKs). RTKs are transmembrane receptors. A subfamily of the RTKs are the neurotrophic tyrosine kinase (Trk) receptors; TrkA, TrkB, and TrkC (Kaplan and Stephens, 1994; Choura and Rebaï, 2011; Basson, 2012). TrkA has a high ligand affinity for NGF (Weismann and de Vos, 2001; Talebian et al., 2013). Upon ligand binding, TrkA homodimerizes and trans-autophosphorylates the tyrosine residues of its cytoplasmic domain allowing proteins to bind the phospho-tyrosine residues leading to the recruitment of adaptor proteins with Src homology 2 (SH2) domains to activate intracellular signaling pathways (Kaplan and Stephens 1994; Cheung and Ip, 2008; Lim and Pawson, 2010). The activity of kinases initiates the phosphorylation cascade of the signaling pathway.

Kinases in Neuronal Development

The most well-characterized neuronal signal transduction pathways are the mitogen activated protein kinase (MAPK) cascade, phosphatidylinositol 3-kinase (PI3K) cascade, and the phospholipase C cascade (Hausott et al., 2009). The MAPK and PI3K cascades are strongly associated with the regulation of neuronal development, whereas
the phospholipase C pathway is mainly involved in neurotrophin release (Canossa et al., 1997; Hausott et al., 2009). It is well characterized that sustained activation of the MAPK cascade induces neuronal differentiation in PC12 cells.

Mitogen Activated Protein Kinase Signaling

The mechanism of MAPK signal transduction is a three-tier phosphorylation cascade of MAPK kinase kinase, MAPK kinase, and MAPK (Figure 1). GTPase Ras activates the three-tiered MAPK cascade. As a GTPase, Ras cycles between an inactive GDP bound form and an active GTP bound form (Wennerberg et al., 2005). These conversions are tightly regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs increase the GDP/GTP exchange rate and GAPs stimulate intrinsic GTPase activity (Bishop and Hall, 2000). Adaptor proteins Grb2 and SOS mediate RTK activation to Ras (Kao et al., 2001). This action occurs at the plasma membrane. Ras is targeted to the membrane due to lipid modifications of its cysteine-aliphatic-aliphatic-X (CAAX) motif (Wennerberg et al., 2005). Ras activates cytoplasmic effectors MAPK kinase kinase, MAPK kinase, and MAPK to induce cellular proliferation, differentiation, and apoptosis dependent on the intensity and duration of the signal response (Rojas et al., 2012). In neuronal cells, it is the activation of MAPK that regulates the transcription of genes for neuronal development.
Figure 1. MAPK and MK-STYX signaling cascade in PC12 cells. Neurotrophins such as NGF induce the homodimerization and trans-autophosphorylation of the receptor tyrosine kinase (RTK). This signal is mediated to the small-G-protein Ras. Ras cycles between an inactive-GDP bound form and an active GTP-bound form. Ras can activate the MAPK phosphorylation cascade. Sustained activation of MAPK, also known as ERK1/2, induces neuronal differentiation in PC12 cells. Mitogen activated phosphatases (MKPs) inactivate MAPKs. Due to shared structural homology with active MKPs, pseudophosphatase MK-STYX may directly affect activation of MAPK, indirectly affect the activity of MKPs, or play a role in a different signal transduction pathway.
Mitogen Activated Protein Kinase

In mammalian cells, the activation of the different families of MAPKs initiates various cellular responses. The main families consist of the extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 kinases (Roskoski, 2012). Each MAPK family is based on different threonine-Xxx-tyrosine motifs (Peng et al., 2010). Ras can activate the signal transduction pathway for all MAPK families. Though the p38 and JNK family kinases primarily play a role in cellular stress responses, p38 and JNK activation has been found to play an important role in morphological changes associated with neuronal differentiation of PC12 cells (Kim et al., 2004; Sarina et al., 2013). However, it is well characterized that the sustained activation of ERK1/2 induces neuronal differentiation in PC12 cells.

Distinguishing it from the other MAPK families, ERK has a threonine-glutamic acid-tyrosine motif (Peng et al., 2010). ERK1 and ERK2 have 84% sequence identity sharing many, if not all, functions (Roskoski, 2012). ERK1/2 is activated by phosphorylation of threonine and tyrosine residues by dual specificity kinase, MAPK kinase (MEK) (Sweatt, 2001). Activation of ERK1/2 leads to the activation of transcription factors important for neuronal survival and plasticity (Sweatt, 2001; Cavanaugh et al., 2001). Temporal regulation of ERK1/2 is very important for the induction of neurite outgrowths. In PC12 cells, transient activation of ERK1/2 only induces cellular proliferation, whereas the sustained activation of ERK 1/2 leads to the induction of neuronal differentiation. This has been demonstrated through cellular
response to epidermal growth factor (EGF) and nerve growth factor (NGF) stimulus. NGF induces the sustained activation of ERK1/2 resulting in PC12 neuronal differentiation whereas EGF induces transient activation of ERK 1/2 resulting in PC12 cellular proliferation (Nakafuku and Kaziro, 1993; Kao et al., 2001). Phosphatases catalyze the inactivation of ERK1/2 therefore regulating sustained or transient activation of ERK 1/2. Therefore, attention must be given to phosphatases as critical regulators of the rate and duration of signal responses.

**Protein Tyrosine Phosphatases in Neuronal Development**

Phosphatases are critical to regulating the phosphorylation cascades initiated by kinases. Unlike kinases which evolved from a common family, phosphatases evolved from structurally and mechanistically distinct families (Tonks, 2006). Protein tyrosine phosphatases (PTPs) are characterized by their catalytic signature motif: His-Cys-X₅-Arg (HCX₅R) (Pannider et al., 1998; Tonks, 2006). PTPs can be divided between classical protein tyrosine phosphatases and dual-specificity phosphatases (Zhang, 2002; Anderson et al., 2004). The cysteine and arginine residues are critical for the PTP catalytic activity. PTPs catalyze protein dephosphorylation with a two step mechanism involving a nucleophilic attack of the phosphate by the sulfur atom of the cysteine residue, and function of the asparagine as a general acid for the protonating of the leaving group (Pannifer et al., 1998; Tonks, 2013). Classical PTPs catalyze the removal of phospho-tyrosine residues, whereas the dual-specificity phosphatases can accommodate both phospho-tyrosine and phospho-serine/phospho-tyrosine residues due to a broader active site (Tonks, 2006; Lim and Pawson, 2010).
PTPs can be positive or negative regulators of neuronal differentiation. Studies have shown that the classical PTP, SHP-2, plays a positive role in the induction of PC12 neuronal differentiation (Wright et al., 1997; Huang et al., 2012), whereas the classical PTP, SHP-1, negatively regulates the survival of neurons (Zhang, 2002). Similar to SHP-2, the dual-specificity phosphatase, PTEN, has been shown to promote neuronal differentiation (Lachyankar et al., 2000). Because prolonged activation of MAPK leads to neuronal differentiation of PC12 cells, this thesis focused research on the mitogen activated phosphatases (MKPs) family which dephosphorylate the MAPKs.

**Dual-specificity Mitogen Activated Phosphatases**

MKPs act antagonistically to the MAP kinases. Mammalian cells have 10 catalytically active MKPs (Dickinson and Keyse, 2006; Caunt and Keyse, 2013). Dual-specificity MKPs have a non-catalytic N-terminal cdc25 homology (CH2) domain and a C-terminal catalytic PTP domain (Tonks, 2006; Owens and Keyse, 2007). The CH2 domain contains a kinase interaction motif (KIM) which mediates the specificity of interaction between the MKPs and the MAPK substrates (Caunt and Keyse, 2013; Tonks, 2013). MKPs can recognize and inactivate a single class of MAP kinases or regulate more than one MAPK pathways (Owens and Keyse, 2007; Staples et al., 2010). MKP-1 and MKP-2 are nuclear phosphatases that have substrate preferences for all three classes of MAPKs (Owens and Keyse, 2007). PC12 cell stimulation with EGF and NGF have been shown to induce an increase of MKP-1 and MKP-2 transcription (Misra-Press et al., 1995). In contrast, in the case of MKP-3, a cytoplasmic phosphatase with a substrate
preference of ERK, transcription levels are increased only when stimulated with NGF in PC12 cells (Camps et al., 1998). MKP-3 expression is sustained for five days, suggesting its role in regulating sustained activation of MAPK induced by NGF stimulation (Camps et al., 1998). MKPs play an important role in the spatial-temporal regulation of MAPKs. However, phosphatases and kinases are not the sole regulators of phosphorylation cascades. Naturally occurring pseudo-enzymes are emerging as critical regulators of phosphorylation cascades.

**Pseudophosphatase MK-STYX**

Pseudo-enzymes are proteins that maintain the ability to bind the substrates of their active homologues but have amino acid substitutions rendering them catalytically inactive. Thus, they cannot catalyze the removal of phosphate groups. The prototype, pseudophosphatase STYX, named as an allusion for the river of underworld, has a glycine substitution for the nucleophilic cysteine rendering STYX catalytically inactive (Wishart et al., 1995). Sequence analysis of the STYX domain shows structural homology with the dual-specificity PTPs (Wishart and Dixon, 1998).

Pseudophosphatases were thought to only function as “dominant-negative proteins” however our investigations strongly suggest that pseudophosphatase MK-STYX [MAPK (mitogen-activated protein kinase) phosphoserine/threonine/tyrosine-binding protein] is a critical regulator of signal transduction cascades.

MK-STYX is a pseudophosphatase member of the MKPs (Wishart et al., 1995; Hinton et al., 2010). MK-STYX is catalytically inactive due to phenylalanine and serine substitutions for critical active residues, histidine and nucleophilic cysteine, respectively.
in the PTP signature motif (Tonks, 2006; Hinton et al., 2010). In the non-catalytic N-terminal domain, MK-STYX has a cdc homology 25 (CH2) motif which is responsible for the specificity of MKP-MAPK interactions (Wishart and Dixon, 1998). MK-STYX is structurally homologous to active MKPs. Point mutations converting the phenyalalanine and serine residues to histidine and cysteine residues, respectively, generates a catalytically functional mutant of MK-STYX (Hinton et al., 2010). As an inactive homolog to MKPs, we investigated the role of MY-STYX in neuronal differentiation of PC12 cells.

**Thesis Objectives**

Sustained activation of MAPK (ERK 1/2) induces neuronal differentiation in PC12 cells. MKPs regulate the duration of MAPK activation. Despite being catalytically inactive, MK-STYX shares structural homology with MKPs suggesting its role in neuronal differentiation of PC12 cells. This thesis investigated the role MK-STYX has in PC12 cell neuronal differentiation. The specific aims were as follows:

1) Does MK-STYX induce differentiation in PC12 cells?

2) Does MK-STYX affect the small GTPases, Ras or Rho?
METHODS

Plasmids

pMT2-FLAG-MK-STYX-FLAG was constructed as described in Hinton et al. (2010). The N-terminal and C-terminal ends of human MK-STYX were flanked by the FLAG epitope for MK-STYX detection. The integrity of the construct was confirmed by sequencing.

Cell Culture

All experiments used rat pheochromocytoma PC12 cells (ATCC). PC12 cells were grown at 37°C and 5% CO₂ in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen) supplemented with 10% horse serum (Invitrogen) and 5% fetal bovine serum (Invitrogen) or Dulbecco’s Modified Eagle Medium [DMEM] (Gibco) supplemented with 10% fetal bovine serum. Cells were maintained with 15 ml per flask (Thermo Scientific) or 5 ml per 60 mm plate (Thermo Scientific). The medium of the cells was changed daily.

Transfection

Twenty-four hours post seeding, transient transfection of PC12 cells was performed using 2 µl Lipofectamine 2000 reagent per 2 µg DNA (Invitrogen). Cells were incubated with transfection reagents for five to six hours before the cell medium was removed and replaced with fresh medium.

MEK Inhibition Treatment

PC12 cells were seeded at a confluency of 1.5×10⁵ cells per well (Fischer). Twenty-four hours post transfection PC12 cells were treated with 50 µM of MEK.
inhibitor, PD980509 (Cell Signaling). Two hours post inhibitor treatment, PC12 cells were stimulated with 100 ng/ml of NGF for three days. Live imaging of PC12 cells was conducted with differential interference contrast microscopy (DIC) and fluorescence microscopy using Nikon Eclipse Ti inverted fluorescence microscopy.

**Time-dependent NGF Stimulation**

Twenty-four hours post-transfection, PC12 cells were serum-starved in RPMI supplemented with 1% horse serum or DMEM with no serum supplementation for approximately 8 to 12 hours. PC12 cells were stimulated with 100 ng/ml of NGF (Prospec) for time points consisting of 0 minutes, 1 minute, 3 minutes, 5 minutes, 12 minutes, 30 minutes, 24 hours, and 48 hours. PC12 cells were promptly lysed after nerve growth factor stimulation.

**Cell Lysis**

PC12 cells were lysed using 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 cocktail tablets [Rochel]) or G-LISA lysis buffer (Cytoskeleton) and protease inhibitor cocktail (Cytoskeleton). On ice, cells were rinsed with 3 ml of cold 1×D-PBS. Cells were lysed and scrapped for approximately 1 minute. For the G-LISA assays, liquid nitrogen was used to snap-freeze the lysates for storage at 80°C.

**Immunoblotting**

Lysates were sonicated and centrifuged at 14,000 rpm for 10 min. Protein concentration was determined using NanoDrop quantification. Lysates were diluted to obtain samples of 50 µg of protein with 6X Laemmli sample loading buffer and
dithiothreitol (DTT). Proteins were separated on a 10% SDS-PAGE for 45 minutes at 150 volts. Protein gels were transferred to a PVDF membrane (GE Healthcare) using Trans-Blot Semi-Dry Transfer Cell (BioRad). Proteins were immunoblotted with anti-STYXL1 (Sigma) or anti-FLAG (Sigma) to detect MK-STYX, anti-β-tubulin (Thermo Scientific) for loading control, and in 5% milk 1X TTBS (Tween 20 Tris Saline Buffer). Protein bands were detected with enhanced chemiluminescence plus (GE Healthcare) and analyzed by autoradiography.

**Fluorescence Microscopy**

For live imaging, cells were plated at a confluency of $1.5 \times 10^5$ cells per well (Fischer). Live imaging of PC12 cells was conducted with differential interference contrast microscopy (DIC) and fluorescence microscopy using Nikon Eclipse Ti inverted fluorescence microscopy.

**Ras G-LISA**

The protocol of the Cytoskeleton Ras G-LISA Activation Assay Biochem Kit was followed using the reagents provided.

Protein concentration was determined using NanoDrop quantification of a 6 µl aliquot of sample prior to snap-freezing. Lysates were thawed in a room temperature water bath then clarified by centrifugation for 1 minute at 4°C and 10,000 rpm. Control protein, blanks, and equalized sample protein concentrations of 0.4 mg/ml to 2.0 mg/ml were added to appropriate wells of the G-LISA plate. The G-LISA plate was incubated on a rotator at 4°C for 30 minutes. The G-LISA plate was washed three times with wash buffer. The G-LISA plate was incubated for 2 minutes with antigen presenting buffer. The
plate was washed three times with wash buffer. The G-LISA plate was then incubated with an anti-Ras primary solution of 1:50 dilution in antibody dilution buffer for 45 minutes at room temperature on a rocker. The primary solution was removed and the G-LISA plate was washed three times with wash buffer. Subsequently, the G-LISA plate was incubated with a horseradish peroxidase secondary solution of 1:250 dilution in antibody dilution buffer for 45 minutes at room temperature on a rocker. The secondary solution was removed and the G-LISA plate was washed three times with wash buffer. The G-LISA plate was then incubated at room temperature with 50 µl of mixed horseradish peroxidase (HRP) detection reagent for 15 minutes. After 15 minutes, 50 µl of HRP Stop Buffer was added to each well. The signal was detected by measuring absorbance at 490 nm using a microplate spectrophotometer.

RhoA G-LISA

The protocol of the Cytoskeleton Inc. RhoA G-LISA Activation Assay Biochem Kit was followed using the reagents provided.

Protein concentration was determined using NanoDrop quantification of a 6 µl aliquot of sample prior to snap-freezing. Lysates were thawed in a room temperature water bath then clarified by centrifugation for 1 minute at 4°C and 10,000 rpm. Control protein, blanks, and equalized sample protein concentrations of 0.4 mg/ml to 2.0 mg/ml were added to appropriate wells of the G-LISA plate. The G-LISA plate was incubated on a rotator at 4°C for 30 minutes. The G-LISA plate was washed three times with wash buffer. The plate was incubated for 2 minutes with antigen presenting buffer. The G-LISA plate was washed three times with wash buffer. The G-LISA plate was then
incubated with an anti-RhoA primary solution of 1:250 dilution in antibody dilution buffer for 45 minutes at room temperature on a rocker. The primary solution was removed and the plate was washed three times with wash buffer. Subsequently, the plate was incubated with a horseradish peroxidase secondary solution of 1:62.5 dilution in antibody dilution buffer for 45 minutes at room temperature on a rocker. The secondary solution was removed and the plate was washed three times with wash buffer. The plate was then incubated at 37°C with 50 µl of mixed horseradish peroxidase (HRP) detection reagent for 15 minutes. After 15 minutes, 50 µl of HRP Stop Buffer was added to each well. The signal was detected by measuring absorbance at 490 nm using a microplate spectrophotometer.

RhoA ELISA

The protocol of the Cytoskeleton Inc. Total RhoA ELISA Kit was followed using the reagents provided and the lysates used in the RhoA G-LISA assay.

RhoA wild-type His-tag positive controls and samples were prepared at a ratio of 1:4 with sample dilution buffer. Diluted RhoA positive controls and samples were added to each well. The ELISA plate was incubated for 2 hours undisturbed at room temperature. After 2 hours, the ELISA plate was washed three times with wash buffer. The ELISA plate was then incubated with antigen presenting buffer at room temperature, undisturbed for 2 minutes. After 2 minutes, the ELISA plate was washed three times with wash buffer. The ELISA plate was next incubated with an anti-RhoA primary solution dilution of 1:2000 in wash buffer for 1 hour at room temperature, undisturbed. After 1 hour, the ELISA plate was washed three times with wash buffer. Subsequently, the
ELISA plate was incubated with a horseradish peroxidase secondary solution dilution 1:300 in wash buffer for 1 hour at room temperature, undisturbed. After 1 hour the ELISA plate was washed five times with wash buffer. The ELISA plate was incubated at room temperature with Horseradish peroxidase detection reagent for 15 minutes. After 15 minutes, a stop solution of 1.8 M sulfuric acid was added to each well. The signal was detected by measuring absorbance at 490 nm using a microplate spectrophotometer.

RESULTS

MK-STYX is endogenously expressed in PC12 cells

It is well characterized that sustained activation of MAPK, also known as ERK1/2, induces neuronal differentiation of PC12 cells. ERK1/2 is activated by serine/threonine and tyrosine residue phosphorylation. MKPs such as MKP1 inactivate ERK1/2 by hydrolysis of the phosphorylated residues. MK-STYX is structurally homologous to the MKPs that regulate the duration of ERK1/2 activation. This suggests that MK-STYX may play a role in neuronal differentiation by sustaining activation of ERK1/2. Therefore, to begin the investigation of MK-STYX in PC12 cells, the presence of endogenous MK-STYX was first confirmed.

Previous studies have detected MK-STYX in human embryonic kidney (HEK) cells (Hinton et al., 2010). This finding was confirmed as a control. PC12 and HEK lysates transfected with MK-STYX and non-transfected PC12 and HEK lysates were immunoblotted with anti-STYXL1 antibody. Over-expression of MK-STYX was also detected by anti-FLAG, the tag to MK-STYX within the pMT2 vector (Hinton et. al,
The presence of endogenous MK-STYX suggests that MK-STYX would be available to serve as a regulator of signal transduction cascades in PC12 cells (Figure 2).

**Figure 2.** MK-STYX is endogenously expressed in PC12 cells. PC12 and HEK cells were transiently transfected with a pMT2-FLAG-MK-STYX-FLAG. Transfected and non-transfected whole cell lysates were lysed and resolved on a 10% SDS-PAGE gel. Immunoblot analysis was performed with anti-STYXL1. (A) Immunoblot analysis confirmed the presence of endogenous and over-expressed MK-STYX in PC12 and HEK cells. (B) To confirm that FLAG-tagged MK-STYX was over-expressed, immunoblot analysis was performed with anti-FLAG.

**MK-STYX induces neuronal differentiation**

The expression of endogenous MK-STYX in PC12 cells suggests it could play a role as a regulator of signal transduction cascades. In order to investigate the effect MK-STYX has on the morphological changes of PC12 cells, MK-STYX was over-expressed. PC12 cells were transfected with pEGFP and pMT2 or pEGFP and pMT2-FLAG-MK-STYX. PC12
cells were scored for the presence of neurite extensions, defined as protrusions greater than 20 µm in length. For each experiment 100 cells were scored (Figure 3). Within 5 days, over-expression of MK-STYX alone was found to induce more cells to have neurite outgrowths. This strongly suggests that MK-STYX induces neuronal differentiation of PC12 cells.
Figure 3. MK-STYX induces neuronal differentiation of PC12 cells. Representative examples are presented to illustrate the growth of neurites in PC12 cells 5 days after transfection (A). Cells were scored for neurite extensions greater than 20µm in length (B). Cells transfected with pMT2-FLAG-MK-STYX-FLAG showed significant induction of neurite outgrowth. This work was performed in collaboration with Kristen Wong. Three replicates of this experiment were performed.
MK-STYX promotes neurite extensions in the absence and presence of NGF

Based on the previous observation that MK-STYX alone was inducing more cells to have neurite outgrowth, we wanted to observe the effects of MK-STYX in nerve growth factor (NGF) stimulated PC12 cells. It is well known that NGF induces the differentiation and neuronal outgrowth of PC12 cells. Previous studies have shown that NGF dose dependently induces neurite outgrowth (Waetzing and Herdegen, 2003). Compared to cells treated with lower concentrations, 50 or 100 ng/ml NGF stimulation of PC12 cells resulted in more cells with neurite outgrowths (Waetzing and Herdegen, 2003).

PC12 cells were treated with 100 ng/ml of NGF to determine if MK-STYX enhanced the effect of NGF stimulation. We found that PC12 cells expressing MK-STYX had longer neurite extensions. These findings suggest that MK-STYX sustains MAPK activity. (Figure 4)
MK-STYX produces neurite extensions in the absence (A) and presence of NGF (B). PC12 cells were plated at a concentration of 1.5 x 10^5 cells. PC12 cells were transfected with pEGFP and pMT2 or pMT2-FLAG -MK-STYX and pEGFP. 24 hours post-transfection, PC12 cells were stimulated with 100 ng/ml of NGF. 48 hours post-transfection, live PC12 cells were imaged using DIC and fluorescence microscopy. NGF induced all of the PC12 cells to differentiate: however, PC12 cells expressing MK-STYX were found to have longer neurite extensions. Three replicates of this experiment was performed.

MK-STYX does not stimulate Ras activation

We have found that MK-STYX induces neuronal outgrowth in PC12 cells, as well as promotes the extension of neuronal outgrowths in the presence of NGF. This leads to the question of the molecular mechanism by which MK-STYX plays a role in PC12 neuronal differentiation and outgrowth.

The Ras-MAPK cascade is a critical component of neuronal differentiation. It is well characterized that NGF binding to TrkA activates a Ras-dependent mitogen activated protein kinase (MAPK) cascade (Nusser et al., 2002). Through this cascade, sustained
activation of ERK1/2 results in the arrest of the growth cycle and stimulation of the differentiation of PC12 cells (Jeon et al., 2012). To begin investigations of the mechanism of action, we studied the activation patterns of the initiator of the MAPK cascade, GTPase Ras. Within minutes of RTK stimulation, Ras is activated leading to the induction of the MAPK pathway (Schiller, 2006). PC12 cells were time-dependently stimulated with NGF to characterize whether MK-STYX induced activation of Ras. PC12 cells were lysed and Ras activation was analyzed using a Ras G-LISA Activation Assay Biochem Kit. Statistical t-test analysis found that MK-STYX caused a significant decrease in Ras activation within 1 minute (Figure 5 and Table 1). However, this decrease was transient and Ras returned to active control levels within 3 minutes. GTPase Ras activates multiple signal transduction pathways. This decrease in Ras inactivation may not affect the activation of the MAPK pathway; however, which lead us to investigation further downstream of the MAPK pathway.
Figure 5. MK-STYX does not stimulate prolonged Ras activation. PC12 cells were transfected with pMT2 or pMT2-FLAG-MK-STYX. 24 hours post-transfection, PC12 cells were serum-starved for approximately 12 hours to bring Ras activity to basal levels. PC12 cells were stimulated with 100 ng/ml of NGF for 1, 3, 5, and 30 minutes. PC12 cells were lysed and activation of Ras was quantified with a Ras GLISA. Three replicates of this experiment were performed. Error bars indicate +/- Standard Error Mean (SEM).

* = p = 0.013 (see Table 1)
Table 1. MK-STYX causes a transient decrease of Ras activation. Statistical two-tailed, equal variant t-test analysis was conducted comparing activation of PC12 cells transfected with pMT2 or MK-STYX at each time point. Statistical analysis showed that MK-STYX induced a transient significant decrease in Ras activation. GTPase Ras is an activator of multiple signal transduction cascades so this inactivation may not affect the activity of the MAPK cascade.

MK-STYX induces neurite growth independent of the MAPK pathway
To determine whether MK-STYX functions through modification of the MAPK pathway, we inhibited the activity of dual-specificity kinase, MEK. MEK induces activation of ERK1/2 through phosphorylation. Sustained activation of ERK1/2 mediates the initiation of neurite outgrowths in PC12 cells. Therefore, inhibition of MEK kinase should inhibit the induction of neurite growths of PC12 cells. The MEK inhibitor prevented neurite outgrowth in PC12 control empty pMT2 vector expressing control cell. However, cells

<table>
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<th>Time point</th>
<th>T-test</th>
<th>Significance</th>
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<tr>
<td>0 minutes</td>
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<tr>
<td>1 minute</td>
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<tr>
<td>30 minutes</td>
<td>0.537</td>
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transfected with MK-STYX maintained expression of neurite outgrowths. This strongly suggests that MK-STYX functions independently of the MAPK cascade (Figure 6).

**Figure 6.** MK-STYX causes neurite outgrowth despite addition of a MEK inhibitor.

PC12 cells were transfected with pMT2-FLAG-MK-STYX. 24hr post-transfection, cells were treated with PD98059 for 2 hr prior to NGF stimulation. Cells were treated with 100 ng/ml NGF and scored for neurites 72 hr after stimulation. Three replicates of this experiment were performed. Error bars indicate +/- Standard Error Mean. This work was performed in collaboration with Kristen Wong.
MK-STYX decreases activation of RhoA

MK-STYX was found to function independently of the MAPK cascade shifting our focus to other pathways involved in neuronal differentiation. PC12 cells grow and proliferate in a relatively round shape. This round morphology must be changed for formation of neurite outgrowths, and involves reorganization of the actin cytoskeleton (Govek et al., 2005). The Rho GTPases are involved in actin cytoskeleton remodeling. Studies utilizing dominant-negative RhoA, shRNA-mediated RNA interference, and Rho-ADP-ribosyltransferase C3 toxin (Clostridium botulinum C3 Rho-ADP-ribosylating exoenzyme), which specifically inactivates Rho isoforms, RhoA, RhoB, and RhoC, have verified that RhoA inactivation is necessary for the induction of neurite outgrowths in PC12 cells (Sebök et al, 1991; Bishop and Hall, 2000; Fan et al., 2008). Similar to the activation of Ras, RhoA responds to receptor tyrosine kinase activation (Schiller, 2006).

We conducted time-dependent NGF stimulation trials to determine if MK-STYX induced a decrease of RhoA activation.

Activation of RhoA was quantified with a RhoA GLISA. RhoA GLISA data were normalized for total RhoA using a RhoA ELISA. PC12 cells expressing MK-STYX had lower levels of RhoA activation. This correlates with studies showing that over-expression of MK-STYX induces neuronal differentiation of PC12 cells (Figure 7).
Figure 7. **MK-STYX decreases RhoA activation.** Inactivation of RhoA is required for the induction of neurite outgrowths in PC12 cells. PC12 cells were transfected with pMT2 or MK-STYX. 24 hours post-transfection, PC12 cells were stimulated with 100 ng/ml NGF at various time points. PC12 cells were lysed, and activation of RhoA was quantified with a RhoA G-LISA. Total RhoA was normalized with a RhoA ELISA. Three replicates of this experiment were performed. Error bars indicate +/- Standard Error Mean.
DISCUSSION

PC12 cell differentiation

The PC12 cell line is a model system for the study of neuronal differentiation. The neurotrophin NGF signals through the Trk receptor inducing sustained activation of ERK1/2. It is well characterized that sustained activation of ERK1/2 causes a cessation of PC12 cellular proliferation and induces neuronal outgrowths. We have shown that MK-STYX plays a critical role in the induction of PC12 neuronal differentiation. We have confirmed that MK-STYX is endogenously expressed in PC12 cells and over-expression of MK-STYX alone can induce neuronal outgrowths of PC12 cells. Furthermore we have found that MK-STYX enhances the effect of NGF by promoting longer neurite outgrowths in PC12 cells stimulated with NGF. This study strongly suggests that MK-STYX plays a role in signal transduction pathways involved in neuronal differentiation and neuronal outgrowth of PC12 cells. MK-STYX is structurally homologous to MKPs. Like MKPs, MK-STYX has a N-terminal CH2 domain for protein-protein interaction and a C-terminal dual-specificity phosphatase domain (Wishart and Dixon, 1998). The structural homology of MKPs and MK-STYX suggests that these proteins share similar substrates and may directly interact with each other. Therefore, we began our investigations with the modulation of the MAPK cascade.

NGF factor stimulation activates a Ras-dependent MAPK cascade leading to sustained activation of ERK1/2 (Nusser et al., 2002). We investigated the activation patterns of the initiator of the MAPK cascade, GTPase Ras. Ras cycles between an active GTP-bound form and inactive GDP-bound form. The action of GEFs and GAPs regulate
Ras activation. Studies have shown, despite NGF stimulation, that dominant-negative Ras blocks neuronal differentiation of PC12 cells; therefore, GTPase Ras is critical for initiating the sustained Ras-dependent MAPK signal transduction cascade for PC12 neuronal differentiation (Nakafuku and Kaziro, 1993). Our time-dependent NGF stimulation studies of PC12 cells found a transient decrease in Ras activation at 1 minute for PC12 cells over-expressing MK-STYX. Ras activation returned to control activation levels within 3 minutes. This finding may seem contradictory of earlier observations that MK-STYX induced neuronal differentiation of PC12 cells. However, Ras is involved in the activation of various signaling pathways involved in cellular proliferation, stress responses, apoptosis, and other cellular responses (Owens and Keyse, 2007). Furthermore, the mammalian subfamily of Ras proteins has various isoforms. These isoforms have a high degree of sequence identity, common downstream effectors, upstream activators, and overlapping functions (Castellano and Santos, 2011). The Ras G-LISA activation assay does not differentiate between the different isoforms of the Ras subfamily. The decrease of Ras activation may have been a Ras isoform not involved in neuronal differentiation. This leads to investigations further downstream MAPK cascade effectors.

MEK is a dual specificity kinase of ERK1/2 (Sweatt, 2001). Inhibition of MEK with PD980589 should cease activation of ERK1/2 (Crews et al. 1992). PC12 cells transfected with the empty pMT2 vector had a significant reduction of PC12 cells expressing neurite outgrowths in the presence of the MEK inhibitor despite stimulation with NGF. However, PC12 cells expressing MK-STYX maintained expression of neurite
outgrowths similar to the control. This strongly suggests that MK-STYX functions independently of the MAPK cascade. Previous studies of MK-STYX siRNAs in HeLa cells found that RNA-silencing of MK-STYX did not have a significant effect on the activation of ERK 1/2 (Niemi et al., 2011). This suggests that MK-STYX does not directly sustain activation of phospho-ERK1/2. Further studies of phospho-ERK1/2 immunoblotting of PC12 cells with MEK inhibition will verify if MK-STYX induces activation of ERK1/2 independent of the MAPK cascade. Furthermore, co-immunoprecipitation analysis can be performed to establish whether ERK1/2 and MK-STYX interact directly, though we hypothesize these proteins do not due to previous studies in HeLa cells (Niemi et al., 2011).

Due to the structural homology with MKPs, MK-STYX may play a role in the modulation of other MAPKs such as JNK and p38 kinases. JNK and p38 kinases are associated with roles in regulation of cellular stress and apoptosis. However, activation of JNK and p38 kinases are critical for neuronal differentiation of PC12 cells (Waetzing and Herdegen, 2003; Sarina et al., 2013). Waetzing and Herdegen suggest that ERK1/2 and JNK synergistically activate NGF-stimulated PC12 cell neuronal differentiation and neurite outgrowth (2003). Their studies suggest that JNKs are responsible for neurite formation, whereas ERK 1/2 is responsible for neurotransmission (Waetzing and Herdegen, 2003). MK-STYX has structural homology with MKP-1 and MKP-2, both of which bind all classes of MAPK (Wishart and Dixon, 1998). Therefore, MK-STYX may play a role in the activation of other MAPKs or effect the activity of the associated MKPs.
Inactivation of RhoA

Within the Ras GTPase superfamily is the Rho subfamily consisting of Rac, Rho, and Cdc25. These highly conserved, eukaryotic GTPases dynamically regulate the actin cytoskeleton morphology. Rho GTPases cycle between an inactive GDP bound form and an active GTP bound form. These conversions are tightly regulated by GEFs and GAPs. Rho GTPases have an additional level of regulation with Rho guanine nucleotide dissociation inhibitors (RhoGDIs), which inhibit the spontaneous exchange of GDP to GTP by isolating GDP-bound Rho in the cytoplasm (Bishop and Hall, 2000).

It is well characterized that RhoA inactivation is required for the induction of neuronal outgrowths in PC12 cells. Studies utilizing dominant-negative RhoA, shRNA-mediated RNA interference, and C3 toxin have verified that RhoA inactivation is necessary for the induction of neurite outgrowths in PC12 cells (Sebők et al, 1991; Bishop and Hall, 2000; Fan et al., 2008). Furthermore, Sebők et al. produced a simple model to verify the different roles of RhoA in PC12 neuronal differentiation. Sebők et al., established that the rate of neurite extension of postmitotic, neuronally differentiated PC12 cells was accelerated by activated RhoA expression, but decelerated by dominant negative RhoA expression (1991).

Our studies found that MK-STYX inactivated RhoA. PC12 cells expressing MK-STYX maintained low levels of RhoA activation compared to control activation levels. Activation of RhoA increased within 48 hours of NGF-stimulation of PC12 cells. This correlates with our live imaging findings that MK-STYX promotes NGF-induced
neuronal outgrowth in PC12 cells. Within 48 hours, PC12 cells seems to shift from inducing neurite outgrowth to extending neurite outgrowth length. RhoA has different roles for the different stages of PC12 neuronal differentiation and outgrowth. RhoA inactivation is required for the induction of neurite outgrowths, whereas activation of RhoA promotes neurite outgrowth extension (Sebők et al., 1991). MK-STYX seems to follow this pattern of activation. We found that MK-STYX alone can induce neuronal differentiation of PC12 cells, explaining the low RhoA activation levels prior to NGF stimulation. We have also visualized PC12 cells with longer neurite outgrowths at 48 hours post-NGF stimulation explaining the increase in RhoA activation. Future investigations of downstream effectors of RhoA such as Rho-associated protein kinase (ROCK) are required to further verify inactivation of RhoA. GTP-bound RhoA disrupts the intramolecular autoinhibitory interactions within ROCK to expose the functional domains (Bishop and Hall, 2000; Nusser et al., 2002).

The role of MK-STYX in the mechanism of RhoA inactivation remains to be determined. Other studies have suggested mechanisms of RhoA inactivation in PC12 neuronal differentiation. Nusser et al. demonstrated that NGF induces protein kinase A (PKA) to mediate phosphorylation of RhoA on serine188 in the C-terminal tail rendering RhoA unable to bind to ROCK (2006). The RhoA and MAPK pathways are not independent signal transduction cascades. These pathways have important mechanisms of crosstalk.
Crosstalk between MAPK and RhoA

Similar to the activation of the Ras-MAPK pathway by RTKs, RhoA GTPases are activated within minutes of RTK stimulation due to Rho GEF mediation (Schiller, 2006). Previous studies have demonstrated that 16 of the 69 known human Rho GEFs are involved in the connection of RTK simulation and Rho GTPase activation (Schiller, 2006). The Rho GEFs may take advantage of Rho GTPase recruitment to the membrane. Rho GTPases have a CAAX (Cysteine-aliphatic-aliphatic-Xxx) motif in the C-terminus that mediates their localization to the membrane (Schiller, 2006). Rho GEF can directly interact with the RTK allowing the quick activation of the Rho GTPase due to their proximity at the membrane (Schiller, 2006). MK-STYX may be involved in inhibition of Rho GEF activity or stimulation of Rho GAP activity.

Summary and Future Directions

This study has initiated investigations of the role of MK-STYX in neuronal differentiation. This thesis provides evidence that over-expression of MK-STYX alone can cause neuronal differentiation, MK-STYX induces neuronal differentiation independent of the MAPK cascade, and MK-STYX causes inactivation of RhoA. These studies implicate a potential therapeutic use of MK-STYX. The disruption of the coordination of protein phosphorylation and dephosphorylation leads to the neurological diseases such as Alzheimer’s disease. Proteins such as Tau have been shown to be hyperphosphorylated in patients diagnosed with Alzheimer’s disease (Hu et al., 2002). Gaining insight on the role of MK-STYX in neuronal differentiation can lead to
future development of MK-STYX as a therapeutic for the treatment of neurological disorders and injuries.

Further studies of MK-STYX in PC12 neuronal differentiation will focus on understanding the mechanism of action of MK-STYX. Phospho-ERK1/2 immunoblotting of PC12 cells with MEK inhibition will be conducted to verify whether MK-STYX induces activation of ERK1/2 independent of the MAPK cascade. Immunoblotting for ROCK will be done to verify inactivation of RhoA. Co-immunoprecipitation analysis will be performed to establish whether MK-STYX interacts directly with MAPKS and MKPs. Finally, MK-STYX knockdown studies will be conducted to determine whether MK-STYX is necessary for PC12 neuronal differentiation. This work was funded by a NSF grant (MCB 1113617 to SDH).
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