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Thrombin-Mediated Signaling Pathways In Neurite Extension and Retraction

by

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The undersigned certify that they have read, and recommend to the faculty of graduate studies for acceptance, a thesis entitled "Thrombin-Mediated Signaling Pathways In Neurite Extension and Retraction" submitted by Rania Mufti in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

Rapid neurite remodeling is fundamental to nervous system development and plasticity. It involves neurite extension that is regulated in a system specific manner by molecules such as NGF which functions through the activation of p44/42 MAPK, p38 MAPK and PI3-K/AKT signaling pathways. It also involves neurite retraction that can be regulated by the serine protease, thrombin. However, the molecular mechanism through which thrombin causes neurite retraction has not been documented. Using the PC12 neuronal cell model, we demonstrate that thrombin utilizes the PI3K/AKT signaling pathway to induce neurite retraction in NGF-differentiated cells. Interestingly, I found that in the presence of thrombin, cells that were still differentiating in response to NGF grew longer neurites compared to those that were exposed to NGF alone. There was also a greater populating cells with longer neurites ($\geq$ 20 μm) when the PC12 cells were simultaneously exposed to NGF and thrombin. Analysis of the signaling pathway through which thrombin enhances NGF-mediated neurite extension in differentiating cells revealed increased and sustained activation of p44/42 MAPK and p38 MAPK. Thus, thrombin elicits opposing effects in differentiated and differentiating neurons through activation of distinct signaling pathways: PI3K/AKT in differentiated neurons, and p44/42 MAPK and p38 MAPK in differentiating neurons. These findings have significant implications specifically in understanding the development of the nervous system, and the

disease processes that afflicts it, including impaired learning and memory, and spinal cord injury all of which require neurite outgrowth.

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List of Abbreviations

AD: Alzheimer disease
BBB: Blood brain barrier
BDNF: Brain-derived neurotrophin factor
BFCN: Basal forebrain cholinergic neurons
cAMP: Cyclic Adenosine Monophosphate
Cdk5: Cyclin-dependant kinase 5
EGF: Epidermal growth factor
ERK: Extracellular signal regulated kinase
GDN: Glia-derived nexin
GDNF: Glial cell line-derived neurotrophic factor
GPCR: G-protein coupled receptor
H/R: Hypoxia/reoxygenation
IGF-1: Insulin-like growth factor
JNK: C-Jun N-terminal kinase
MAPK: Mitogen-activated protein kinase
NGF: Nerve growth factor
NTF: Neurotrophic factors
PAR: Protease activated receptors
PC12: Pheochromocytoma cells
PCR: Polymerase chain reaction
PD: Parkinson's disease
PDK: Phosphoinoside-dependent kinase
PG-J: Prostaglandin-J
PI3K: Phosphatidylinositol-3 kinase
PKA: Protein kinase A
PLC: Phospholipase C
PN-1: Protease Nexin-1
PtdOH: Phosphotidyl inositol
SAPK: Stress-activated protein kinase
TGF: Transforming growth factor
TNF: Tumor necrosis factor

TRAP: Thrombin receptor activating peptide

TrkA: Tyrosine kinase A receptor

VEGF: Vascular endothelial growth factor

Chapter 1. Introduction, Hypothesis and Objective

1. Introduction

1.1. Neurotrophic factors

Neurotrophic factors (NTFs), also known as neurotrophins, are a family of proteins that acts as growth factors for development and maintenance of specific neuronal populations in the nervous system. They promote neuronal survival by preventing the initiation of apoptosis and stimulating axonal growth. They also induce differentiation, and play a role in modifying neuronal dysfunction and astrocyte activation that in turn provide neurons with nutrients. Thus, NTFs factors are important in the maintenance, and protection of both normal, and injured neurons (Siegel and Chauhan, 2000). Specifically, NTFs have been shown to play a significant role in the promotion of axonal regeneration (Lykissas, Batistatou, et al, 2007; Siegel, and Chauhan, 2000).

There are different members of neurotrophic factors family: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT4/5). Neuropoietic cytokines, fibroblast growth factor (FGF-2), transforming growth factor (TGF), and insulin-like growth factors (IGF-1) (IGF-2) also play a role in neuronal growth and stimulation (Lykissas, Batistatou, et al, 2007; Siegel, and Chauhan, 2000). Neurotrophins exist *in vivo* as homodimers, previous studies have shown heterodimeric neutrophins, these neurotrophins

have been shown to trigger differentiation in dorsal rat ganglia (DRG) sensory neurons that express multiple Trk receptors (Treanor, Schmelzer, et al, 1995).

Epidermal growth factor (EGF) is one of the growth factors that plays an important role in the regulation of cell growth, proliferation, and differentiation. However, EGF alone cannot cause cell differentiation, only when cyclin adenosine monophosphate (cAMP) is present; neurite extension in PC12 cells is synergistically induced. On the other hand, NGF can independently cause cell differentiation and survival through the TrkA receptor, without the use of cAMP (Lambeng, Michel, et al, 2001). Treating embryos or postnatal animals with NGF can rescue most of neurons that normally die. If a neuron is unable to obtain a sufficient quantity of neurotrophic factors, it will die. This cell death may be due to the inability of the cell to gain the appropriate amount of neurotrophic molecules, whereas the exogenous supplies of trophic molecules may rescue the neurons (Oppenheim, 1989). Previous studies have demonstrated the existence of endogenous NGF receptor molecule in rat brain by immunoprecipitation of its cortical extracts (Arai, Miklossy, et al, 2006).

1.1.1. Neurotrophin receptors

Each member of the neurotrophin family binds to their homologous tyrosine kinase receptors or family of receptors (Martin-Zanca, Oskam, et al, 1989). NTFs

activate two different receptors; tyrosine kinase receptors (Trk), and p75 a member of the tumor necrosis factor (TNF) receptor family. Trk receptors are known to mediate different signaling in different cell types; for example: it promotes apoptotic pathways in tumor cells. Mitosis and differentiation pathways have been also identified as a result of Trk receptors activation. The Trk receptors consist of three classes: TrkA, TrkB, and TrkC. TrkC has a modified kinase domain, while TrkA and TrkB lack the kinase domain (Patapoutian, and Reichardt, 2001). NGF binds to TrkA, while BDNF and NT-4 bind to TrkB, and NT-3 binds to TrkC (Lee, Rajagopal, and Chao, 2002). NGF is released from the target cells, binds to and activates its high affinity receptor TrkA, which is subsequently internalized into the responsive neuron.

Neuronal survival and neurite outgrowth are the main roles of the TrkA receptor (Andjelkovic, Suidan, et al, 1998). After a neurotrophin binds to a Trk receptor, dimerization and kinase activation occur (Lykissas, Batistatou, et al, 2007; Patapoutian, and Reichardt, 2001). After binding and activating TrkA, NGF triggers a signaling cascade that is important for survival, differentiation, and maintenance of neurons (Delcroix, Valletta, et al, 2004).Trk receptors not only play an important role in the central and peripheral nervous systems, but also involved in non-neuronal tissues, which means that NGF mediated effects are not restricted specifically to the nervous system (Nico, Mangieri, et al, 2008). In neonatal rat brain, $p75^{NTR}$ has been shown to mediate apoptosis in neuronal cells

(Andjelkovic, Suidan, et al, 1998). When NGF binds to the $p75^{NTR}$ receptor, it leads to cell death, but if NGF binds to TrkA, apoptosis is prevented.

Trk receptors regulate synaptic strength and plasticity in the mammalian nervous system, which affect neuronal survival and differentiation through several signaling cascades. Trk receptors activate PI3K through two different pathways, whose importance differs between neuronal subpopulations: Ras dependant activation of PI3K through neutrophin mediated cell survival, and by the three adaptor proteins: Shc, Grb-2 and Gab-1. (Patapoutian, Reichardt 2001). The two major effectors pathways for neurotrophin and Ras activated survival are; PI3K/AKT and MEK/MAPK (Kaplan, Miller 2000).

Another receptor family is the G-protein coupled receptors (GPCRs), also known as seven transmembrane domain receptors. GPCR and TrkA are both able to initiate mitogen activated protein kinases signaling pathways. Specifically, the ERK (p44/42 MAPK), JNK/SAPK, and p38 MAPK signaling pathways are activated through GPCRs. EGF and platelet derived growth factor (PDGF) are trans-activated through GPCRs. It is well established that neurotrophins utilize Trk receptors, but recently it has been shown that the activation of Trk receptors can be through GPCRs. The trans-activation of the GPCRs by many neurotrophins such as EGF and NGF, trigger the activation of MEK1, PI3K, and PKA/C does not affect Trk receptor activity.

1.1.2. Mode of neurotrophin action

TrkA receptor tyrosine kinase is a high affinity catalytic receptor for NGF, and signaling through TrkA is central to NGF action. NGF binding causes dimerization and autophosphorylation of TrkA receptors at their tyrosine residues, resulting in the formation of docking sites for SH2 domain containing proteins (Pawson, and Nash, 2000). This results in the activation of Rap1 (GTP-binding protein) and Raf (protein kinase), and cause subsequent activation of the signaling cascades that include p38 MAPK and ERK, phosphatidylinositol-3 kinase (PI3K/AKT), and phospholipase C pathways (Kaplan, and Miller, 2000; Patapoutian, and Reichardt, 2001). There are ten tyrosines in the cytoplasmic domain of each receptor, and phosphorylation of these specific amino acids causes a cascade of kinase activation and phosphorylation in pathways such as the: ERK, PI3K and PLCγ pathways (Patapoutian, and Reichardt, 2001). In addition to their involvement in neurite outgrowth, trophic factor can also affect many aspects of neuronal function and they range from ion channel modulation to synaptic plasticity, underlying learning and memory (Lee, Rajagopal, and Chao, 2002).

1.1.3. Role of NGF in the nervous system

Regulation of neurite extension and retraction is required for normal synaptic connection and signaling, which is important for the establishment of neuronal networks during development (Toman, R. E. 2004). NGF has been shown to play

a role in survival, both *in vivo* and *in vitro*, differentiation through extracellular factors, and the normal function of sympathetic neurons (Katzir, Shani, et al, 2003; Toman, Payne, et al, 2004). The addition of NGF to serum starved PC12 cells shows no visible changes in proliferation, but promotes neurite outgrowth. This illustrates NGF's dual role in the neuron-like cells: mitogenic and anti-mitogenic activity. The mitogenic activity is due to NGF stimulation and subsequent proliferation, for example: melanoma, neuroblastoma and pheochromocytoma. While the anti-mitogenic activity manifests as suppressed cell division, and the stimulation of neuronal differentiation (Burstein, and Greene, 1982; Patapoutian, and Reichardt, 2001).

Other studies have indicated that when NGF stimulates $p75^{NTR}$, it is dependent on the expression of TrkA, if TrkA is not expressed a pro-apoptotic effect is seen. If TrkA is present however, neuronal growth is promoted and also synergistic activation of the AKT survival factor occurs (Kaplan, and Miller, 2000; Miller, and Kaplan, 2001). The $P75^{NTR}$ receptor increases ceramide and activates the JNK/p53/Bax cell death pathway, as well as cdc42/Rac1, Ask1/MEKK1, MKK4/7, JNK and c-jun (Kaplan, and Miller, 2000). These receptors play an important role not only in the differentiation of tissues, but also for nervous system differentiation (Patapoutian, and Reichardt, 2001).

Because the different effects of various NTFs on neurons can be studied directly, many labs have opted to use PC12 cells model for their studies. PC12 cells originate from a tumor of rat adrenal medulla at the same neural crest origin as

certain types of neurons. After NGF triggers PC12 cells growth, a redistribution of actin filaments occurs and promotes neurite extension (Lykissas, Batistatou, et al, 2007). Thus this cell line shares many similar properties with neuronal cells, especially upon differentiation. Monitoring growth cone behavior in vertebrate brain is a very complex task, but PC12 cells provide a simple model for that task. PC12 cells are a useful model for studying neuronal cell signaling such as neuronal migration, synaptic plasticity and development of human nervous system (Robertson, A. 2001). They have distinct responses to differentiation, proliferation, and survival which can be assessed independently (Yang, Liu, et al, 2006). PC12 cells differentiate and form neurites upon exposure to NGF, but when NGF is removed, they de-differentiate and return to their original proliferative state (Burstein, and Greene, 1982). After differentiating PC12 cells with NGF, the cells leave the cell cycle at the G1 growth phase. Thus, NGF acts as a progression factor on cells that are in S (synthesis) phase, or G2 growth phase allowing them to complete the cell cycle before arresting the cells in G1.

Various growth factors are affected by Cyclin dependant kinase 5 (Cdk5). Cdk5 has been shown to play an essential role in the development of the nervous system, including neuronal migration and neurite outgrowth. Cdk5 associated kinase activity has been demonstrated only in mammalian brains. NGF causes sustained activation of p35, the neuron specific activator of Cdk5 through the ERK pathway, which is also required for neuronal differentiation. Cdk5 promotes neuronal survival by phosphorylation and activation of PI3-K survival pathway

(Zheng, Li, et al, 2007). Roscovitine, a Cdk5 inhibitor, when added to NGF treated cells, blocks neurite outgrowth (Harada, Morooka, et al, 2001).

1.1.4. Role of NGF in neurodegenerative diseases

Recent studies have shown that decreases in the neurotrophin levels in different neuropathologic diseases are due to age, genetics, neurodegeneration, or any combination of those. Thus, it has been suggested that the loss of endogenous neurotrophic factors causes Alzheimer's and Parkinson's diseases (Lykissas, Batistatou, et al, 2007).

In the past years, the role of neurotrophic factors has been at the forefront of therapy for neurological disorders. There is little known about the distribution of neurotrophic factors, and their regulation in normal and diseased brain tissue (Siegel, and Chauhan, 2000). Alzheimer's disease (AD) is characterized by the accumulation of β-amyloid and neurofibrillary tangles (Siegel, and Chauhan, 2000). In AD brain, increases in NGF and decreases in BDNF have been discovered. NGF has been shown to prevent degeneration of basal forebrain cholinergic neurons (BFCN), which is a cause of neuronal cell death. Moreover, NGF appears to be the most effective molecule for the survival and maintenance of cholinergic neurons. Thus, NGF may be considered a therapeutic agent for AD patients (Siegel, and Chauhan, 2000). Another neurodegenerative disorder Parkinson's disease (PD), is characterized by a degeneration of nigral

dopaminergic neurons, and depletion of striatal dopamine. In PD brain, previous data showed decreases in the neuronal content of glial cell derived neurotrophic factor (GDNF) and FGF in surviving substantia nigra (SN) dopaminergic neurons.

BDNF level was measured by ELISA in portions of SN, striatum and cerebellum in PD brains and was significantly reduced in SN and striatum of PD human adult brain. Also, NGF was measured by ELISA and was shown to be reduced in SN of PD brains (Siegel, and Chauhan, 2000). However, neurotrophic factors are not only important for development and survival; they also have a role in cognition, learning and memory. Thus, proper regulation and signaling is essential for normal neuronal activity. In Alzheimer's disease for example, NTFs are dysregulated and axonal transport is impaired. NGF has an important role in the development of cholinergic neurons, which are involved in learning and memory. Previous studies have demonstrated that NGF is able to increase memory deficit in AD mice when they are treated with NGF intranasally (De Rosa, Garcia, et al, 2005). In schizophrenia patients, it was observed that the NGF, NT-3, and BDNF levels were not properly regulated, with studies showing either lower or higher levels than normal affect the development of cholinergic fibers. Following systematic administration of NGF, the plasticity and function of the sympathetic nervous system is restored, and a reduction in proliferation of neurogenic tumor cells is seen, as well as beneficial effects in human peripheral neuropathies (Tria, Fusco, et al, 1994). Therefore, the level of specific neurotrophins can serve as a molecular marker of abnormal nervous system development (Shoval, and Weizman, 2005). NGF may be a useful molecular marker and potential

therapeutic agent for many of these neurodegenerative diseases. Together the above studies underscore the importance of NGF not only during development but also its role as therapeutic agent in neurodegenerative disease.

1.2. Mitogen-activated protein kinase (MAPK)

MAPKs play a central role in rapid neurite remodeling which is important for human nervous system development and plasticity including neurite extension and retraction. MAPKs are signaling serine/threonine kinases that are activated by dual threonine/tyrosine phosphorylation in response to extracellular stimuli, they have an important role in growth and differentiation (Lee, Johnson, et al, 2001). The MAPKs family includes: extracellular regulated kinase (ERK), p38 and JNK. The ERK and the classical MAP kinases are widely expressed protein kinase intracellular signalling molecules which are involved in the regulation of meiosis, mitosis, and differentiated cells.

Threonine and tyrosine phosphorylation activates both ERK1/2, at Thr202/Tyr204 for human ERK1 and Thr185/Tyr187 for human ERK2. Unlike MEK, significant ERK activation requires phosphorylation at both sites, with tyrosine phosphorylation preceding that of threonine. ERK1 is also known as mitogen activated protein kinase 3 (MAPK3), ERK2 as MAPK1, ERK3 as MAPK6, and ERK4 as MAPK4. ERK3 and ERK4 are analogues which show major differences only in the C-terminal extension. ERK5, also known as MAPK7, is a recently discovered protein. ERK 7/8 (MAPK15) is a novel diagnostic cancer marker

which includes a long C terminus similar to ERK3/4 (Bogoyevitch, and Court, 2004).

The p44/42 MAPKs (ERK1/2), the most widely expressed members of the MAPK family, can be activated by growth factor receptors (Robinson, and Dickenson, 2001). P44/42 is an important mediator of signal transduction and plays a role in many cellular processes such as cell growth, proliferation, differentiation, and apoptosis (Grewal, York, and Stork, 1999). Activated ERK1/2 is translocated to the nucleus where it phosphorylates several transcription factors, inducing neuronal differentiation (Harada, Morooka, et al, 2001). In NGF-treated PC12 cells, sustained ERK activation is essential for neuronal differentiation. When ERK and p38 inhibitors are used on PC12 cells after NGF stimulation, suppression of transcription factors such as: AP-1, c-jun, and c-Fos illustrate the importance of this pathway on neuronal differentiation (Eriksson, Taskinen, and Leppa, 2007).

There are also four isoforms of p38 MAP kinase, p38-α (MAPK14), p38-β (MAPK11), p38-γ (MAPK12 or ERK6), and p38-δ (MAPK13 or SAPK4). These proteins are involved in cellular differentiation and apoptosis. MAPK is activated by dual phosphorylation on Thr180/Tyr182. The isoforms of p38 kinases are differentially activated by MKK3 and MKK6.Activation of the p38 MAP kinase pathway occurs due to phosphorylation at Thr180/Tyr182. P38 MAPK is widely distributed in many areas; olfactory tract, corpus callosum, cingulum, internal capsule, hippocampi, and stria medullaris

The c-Jun N-terminal kinases (JNKs), include MAPK8, MAPK9, MAPK10, and are also known as stress-activated protein kinases (SAPKs). There are three isoforms of JNK. JNK1 and JNK2 are ubiquitously distributed. By contrast, JNK3 is found mainly in neuronal tissue and testes. JNK1 and/or JNK2 must have a role in both apoptosis and the immune response.

High expression and activity of JNK1 and p38 MAPK has been shown in rat brain (Mielke, Brecht, et al, 1999). P38 MAPKs are activated in PC12 cells after the addition of NGF, although by removing NGF, p38 activation is increased following subsequent apoptosis. Activation of p38 MAPK in PC12 cells is important to stimulate the bone morphogenetic protein (BMP-2) which induces neuronal differentiation. Activation of the ERK and p38 pathway requires full induction of neurite outgrowth after the addition of NGF to PC12 cells (Iwasaki, Iguchi, et al, 1999). Also, p38 MAP kinase in conjunction with the AP-1 signaling pathway is important in promoting the activity of 15-deoxy-prostaglandin J_2 (15-deoxy-PGJ_2), 15-Deoxy-Delta and PGJ_2 resulting in the differentiation of PC-12 cells (Jung, Park, et al, 2003).

NGF also exerts a neuroprotective effect on PC12 cells. In serum-starved cells; NGF-induced activation of the PI3-K/AKT pathway is responsible for the cell survival in *vitro* and *in vivo*. This comprises three highly homologous members known as PKB-α (AKT1), PKB-β (AKT2) and PKB-γ (AKT3). The translocation of PKB/AKT to the plasma membrane, it is activated by phosphorylation upstream kinases including the phosphoinoside-dependent kinase 1 (PDK1). Key roles for

this enzyme can be found in cellular processes such as, cell proliferation, apoptosis, transcription ,and cell migration (Andjelkovic, Suidan, et al, 1998). These studies also described NGF stimulated PKBγ as having the highest activity, and NGF stimulated PKBα activity to be much higher than that of PKBβ (Andjelkovic, Suidan, et al, 1998).

1.2.1 Role of MAPK in the nervous system

In serum-starved PC12 cell cultures, NGF binds to the TrkA receptor, subsequently activating MAPK signaling pathways and cell differentiation (Morooka, and Nishida, 1998; Yaka, Gamliel, et al, 1998). Activation of AKT, ERK1/2, and p38 are essential for PC12 cell differentiation (Kim, Seger, et al, 2004). Previous studies have shown in cerebellar, cortical and motor neurons, PI-3K accounts for 80% of NGF induced survival of the neurons (Kaplan, and Miller, 2000). Over expression of AKT is actually a negative regulator of NGF inducing neurite extension (Bang, Park, et al, 2001). However, another study showed by using mutant growth factor receptor lacking the PI3K binding site that the presence of PI3K in neuronal differentiation is not essential (Obermeier, Bradshaw, et al, 1994). These results may be due to the total cellular activity of AKT in different cell lines, each cell line has different susceptibility to the growth arresting effect of NGF (Bang, Park, et al, 2001).

NGF also causes activation of $p21^{Ras}$ in neuroblastoma cell lines, and thus has a role in signal transduction pathways. When NGF causes activation of $p21^{Ras}$, it is

dose dependant, and is associated with neuroblastoma cell differentiation (Burchill, Berry, and Lewis, 1995). In signaling pathways resulting in neurite extension, not only the Ras/Raf/MEK/ERK pathway is involved, also the p38/MAPK, and PI3K/AKT pathways play a role (Burry, 2001). They are involved in cell proliferation, survival, and migration in some tissues. It is important to note that the $p75^{NTR}$ is also stimulated by NGF and contributes to the subsequent TrkA phosphorylation and activation of the MAPK pathways (Perrone, Paladino, et al, 2005).

1.2.2. MAPK inhibitors

The previous studies showed the importance of the MAPKs signaling pathways in the development of human nervous system. MAPK inhibitors are utilized in order to emphasize the role of the specific inhibited MAPK. For example: EGF is involved in MAPK pathway differentiation; it causes transient activation, while NGF causes sustained activation. By using MEK inhibitor, PD184352, after stimulation of the cells with NGF, a loss in Raf-1 activity was seen, illustrating that NGF is a positive regulator, while EGF is a negative regulator of the pathway (Santos, Verveer, and Bastiaens, 2007). Previous studies have shown that PC12 cell treated with NGF induce phosphorylation and activation of p38, as well as sustained activation of p44/42 MAPK. SB203580, a specific inhibitor of p38 MAPK, causes a marked inhibition of neurite outgrowth in PC12 cells. However, by inhibiting MEK1/2, which is upstream of p44/42 in the pathway, with the

inhibitor U0126, neurite outgrowth is not entirely inhibited. This indicates that p38 MAPK is required for PC12 cell differentiation (Jung, Park, et al, 2003; Lee, Johnson, et al, 2001; Morooka, and Nishida, 1998). P38 inhibitors SB203580 and SB202190 were used for chick embryonic neurons *in vitro*, induces survival of the sensory, sympathetic, ciliary and motor neurons (Horstmann, Kahle, and Borasio, 1998). Other studies confirm that p38 causes cell apoptosis by showing that cleaving Xenopus embryos injected p38 forces the embryo into mitotic arrest (Takenaka, Moriguchi, and Nishida, 1998).

There are several studies implicating the involvement of p44/42 MAPK in PC12 cell differentiation. Phosphorylated p44/42 MAPK is translocated to the nucleus where it activates transcription factors, such as c-Fos (Grewal, York, and Stork, 1999; Lee, Johnson, et al, 2001), which regulates cell differentiation. By inhibiting MEK, upstream of ERK1/2, inhibition of c-Fos and cyclin D1 have been observed (Lee, Johnson, et al, 2001). The ERK signaling pathways have many roles in activity dependent regulation of neuronal function. These pathways integrate second messenger systems, such as protein kinase A (PKA) that regulate ERK signaling and therefore neuronal survival and synaptic plasticity (Grewal, York, and Stork, 1999). Mutagenized PC12 cells have been found to be incapable of differentiation and show only transient activation of p44/42 MAPK after exposure to NGF (Yaka, Gamliel, et al, 1998). Furthermore, sustained activation of p44/42 MAPK is inhibited in PC12 cells treated with anandamide, or endocannabinoid. These molecules inhibit differentiation in NGF treated cells by lowering the potency of NGF and therefore causing a decrease in Raf1/B-Raf mediated ERK

activation. Moreover cannabinoids, which act as neuromodulators, attenuate the effects of many signaling molecules such as the MAPK family (ERK, P38, and JNK), and protein kinase B (Rueda, Navarro, et al, 2002). The canabinoid receptors, CB1 and CB2 are located in the brain and non-neuronal cells, respectively. CB1 inhibits glutamatergic neurotransmission in the substantia nigra pars reticulate. The subthalamonigral pathway inhibition caused by the canabinoid molecules may be important for future treatment of Parkinson's disease (Szabo, Wallmichrath, et al, 2000).

It is well known, that AKT is involved in cellular survival pathways, by inhibiting apoptotic processes. AKT modulates the mitochondrial membrane potential (Liot, Gabriel, et al, 2004) and phosphorylation of its downstream components results in the blockade of apoptosis (Brunet, Datta, and Greenberg, 2001). Also, activation of the AKT pathway inhibits apoptosis through the phosphorylation and inactivation of pro-apoptotic Bcl-2, a member or the BAD family, or caspase-9. This has been previously proven by using dominant negative AKT from rat hearts, leading to blockage of Bcl-2 expression (Uchiyama, Engelman, et al, 2004). By using the MEK1/2 inhibitor, U0126, together with the PI3-K/AKT inhibitor, LY29400, there was an overall reduction in the anti-apoptotic effect, although the MEK1/2 inhibitor failed to prevent the neuro-protective activity of NT-3 (Liot, Gabriel, et al, 2004). PI3-K and AKT are found in the cytoplasm, but are translocated to the nucleus after the administration of growth factor treatment to the cell (Xuan Nguyen, Choi, et al, 2006). Overexpression of AKT will inhibit hypoxia/reoxygenation (H/R) induced caspase-3 and caspase-9 activation, and

cytochrome C release. In addition to AKT pathway for promoting neuronal survival, the Ras-Raf-MAPK-RSK pathway also has a crucial role in neuronal survival (Brunet, Datta, and Greenberg, 2001). The AKT inhibitor, LY294002, blocks NGF, it prevents the induction of AKT phosphorylation, as well as neurite outgrowth, which means PI3K/AKT is positively regulated by NGF to induce neuronal differentiation in PC12 cells. Indeed, activation of AKT, ERK1/2, and p38 is essential for PC12 cell differentiation (Kim, Seger, et al, 2004). It was found that over expression of AKT is actually a negative regulator of NGF inducing neurite extension (Bang, Park, et al, 2001). These results may be due to the total cellular activity of AKT in different cell lines, each cell line has different susceptibility to the growth arresting effect of NGF (Bang, Park, et al, 2001).

1.3. Thrombin

Growth factors are known for their involvement in the development and maintenance of specific neuronal populations in the nervous system. But there are other extracellular molecules share those properties such as thrombin. Thrombin is a serine protease, which is part of the class of enzymes that cut peptide bonds in proteins. This activity depends on a set of amino acid residues in the active site of the enzyme. Thrombin is well known for its role as a coagulation protein, as well as for regulating hemostasis and thrombosis. Also, it is released immediately into the brain after injury, such as brain trauma, blood/brain barrier breakdown, or intracerebral hemorrhage (Jiang, Wu, et al,

2002). Thrombin is a 36kDa serine protease comprised of two chains: A and B. The A chain is comprised of a 36 amino acid residue, while the B chain consists of 259 amino acids. Serine proteases and serpins have been shown to have essential roles in the nervous system (Turgeon, and Houenou, 1997). Prothrombin, a thrombin precursor, converts fibrinogen into insoluble fibrin, which is important in the formation of protective clots. Both prothrombin and thrombin are expressed in the olfactory bulb, thalamus, cortex and corpus striatum of the brain (Dihanich, Kaser, et al, 1991; Turgeon, and Houenou, 1997). By polymerase chain reaction (PCR) and northern blot analysis, the level of prothrombin mRNA was found to be low in neonatal rat brain, but increased in the adult brain. Thrombin plays an essential role in brain development. The genes encoding prothrombin, thrombin, thrombin receptors, and protease inhibitors have been discovered in many areas of the brain (Festoff, Smirnova, et al, 1996).

Thrombin is secreted by the growth cone of developing axons, and degrades the extracellular matrix in order for the growth cone to reach its target (Pittman, 1989). However, axonal targets secrete serine protease inhibitors in response to axonal contact, causing inhibition of axonal outgrowth (Dennis, 1981; Turgeon, and Houenou, 1997). Interplay between thrombin and neurotrophic factors is necessary for proper nervous system development. Thrombin can induce glial cell proliferation and is able to exert both protective and toxic effects on neurons (Nicole , Goldshmidt, et al, 2005; Smith-Swintosky, 1997). Previous studies have

identified the domains in thrombin that are responsible for neurite retraction activity, β and γ derivatives, which are altered in that they contain a positively charged surface, the anion-binding site (Suidan, Stone, et al, 1992). However, it was also discovered that thrombin confers a neuroprotective effect *in vivo* in response to oxidative stress or hypoglycemia (Rohatgi, Sedehizade, et al, 2004). Activation of thrombin in brain cells causes cell tolerance through activation of the thrombin receptor and p44/42 MAPK pathway, and thus confers a neuroprotective effect. For example: low doses of thrombin attenuate edema. Further *in vitro* studies have shown that low doses of thrombin (1-2U/ml) did not cause cell death, while concentrations higher than 5U/ml causes lactate dehydrogenase release leading to death of the cells. By using PD98059, a MEK inhibitor, the neuroprotective effects of thrombin are blocked (Jiang, Wu, et al, 2002).

Also it has been found that thrombin increases the level of acetylcholine transferase in the cells, which plays a prominent role in the nervous system. The great importance of acetylcholine derives from its role as a neurotransmitter for cholinergic neurons, which innervate many tissues, including smooth and skeletal muscle, the heart, and ganglia. The effect of stimulating a cholinergic nerve, for example the contraction of skeletal muscle or the slowing of the heartbeat, results from the release of acetylcholine from the nerve endings (Suidan, Niclou, and Monard, 1996). Also, it is mitogenic to fibroblasts and astrocytes, and contributes to neurite outgrowth. Astrocytes are crucial for the maintenance of

brain function, such as the uptake of ions, neurotransmitters, and also in the formation of tight junctions that form the blood brain barrier. In addition, they synthesize and secret neurotrophic factors that will provide trophic support for differentiation of cell populations. Previous studies have shown astrocytes actively grow in serum or thrombin containing media, but when thrombin concentration is increased, neurite retraction and rounding of the cells occur. The disruption of the astrocyte morphology leads to damage of other brain tissues, such as the blood brain barrier (Turgeon, and Houenou, 1997). These effects can be prevented by using thrombin inhibitors.

1.3.1. Thrombin receptors

Thrombin-related cellular effects in the neural systems are initiated by binding of protease activated receptors (PARs). These cell surface receptors belong to the G protein-coupled protease activated receptor family, that regulate cellular response to extracellular serine protease such as thrombin, trypsin and tryptase; which stimulate cells and causes cell proliferation (Freedman, 2001; Luo, Wang, and Reiser, 2007; Wang, and Reiser, 2003). Thrombin binds to PAR-1, PAR-3, and PAR-4, while PAR-2 is activated by trypsin, an intestinal digestive enzyme. (Wang, and Reiser, 2003).

Previous study showed, only PAR-1 and PAR-2 were identified in human brain, suggesting that PAR-1 is the relevant form in thrombin-mediated effects in brain

(Rohatgi, Sedehizade, et al, 2004; Wang, and Reiser, 2003). In contrast, a study by (Striggow, Riek-Burchardt, et al, 2001) shows that PAR1- 4 are present in the brain using immunohistochemical analysis. They are involved in different roles such as cellular death and survival. PAR-1 and PAR-2 are involved in phosphorylating ERK1/2 and p38 MAPK (Vergnolle, Ferazzini, et al, 2003; Wang, Luo, et al, 2006). PAR-4 is required in higher concentrations to activate thrombin mediated cascade than do the other PARs (Luo, Wang, and Reiser, 2007).

Thrombin and PAR-1 promote mitogenic effects via ERK1/2, PI3K and PLC/Ca2+/PKC. Previous studies have described TRAPs, thrombin receptor activating peptides, as having the ability to reproduce the effect of thrombin by stimulating DNA synthesis (Reilly, Connolly, et al, 1993). In contrast, another study showed that TRAP alone is not sufficient to cause DNA synthesis (Vouret-Craviari, Van Obberghen-Schilling, et al, 1992). Astrocytes proliferation depends upon PAR-1 activation that activates tyrosine kinase activity. On the other hand, in 132 1N1 astrocytoma cells, there is no role for thrombin receptor in DNA synthesis. This controversy is maybe due to the fact that different cell types were used. PAR-1 is the most prominent in the PARs family for causing proliferation effects. Also, thrombin caused activation of p38 MAPK and c-Jun in microglia cells (Wang, and Reiser, 2003). On the other hand, continued thrombin exposure caused cell apoptosis due to the over activation of PAR1, by activating apoptotic pathway via caspase-3 and caspase-1 (Wang, and Reiser, 2003).

1.3.2. Thrombin receptor agonist peptides

Thrombin can cleave PAR-1; this cleavage generates a new N-terminus that functions as a tethered ligand domain: SFLLRN. It is a subclass of GPCR (Hollenberg, and Compton, 2002). The tethered ligand sequence, SFLLRN, was shown to activate PAR1 and PAR2. TRAP are synthetic peptides of five or more residues that act like the new amino-terminus, and activate the thrombin receptor PARs (Chen, Ishii, et al, 1994; Gerszten, Chen, et al, 1994; Grabham, and Cunningham, 1995). Free SFLLRN activates PAR1 independent of receptor cleavage, and has been used to probe PAR1 function in various cells and tissues (Hammes, and Coughlin, 1999). By reversing the first two amino acids in the TRAP sequence: FSLLRNP, this peptide was used as the control and showed little effect (Suidan, Stone, et al, 1992). On the other hand, the agonist peptide does not activate PAR1 in the same manner as thrombin (McLaughlin, Shen, et al, 2005).

In astrocytes, SFLLRNP stimulated 90% of cells, increasing cell number and DNA synthesis. Additionally, other studies have shown that tyrosine kinase activity plays a role in thrombin and TRAP signaling pathways when stimulates cell proliferation. By using herbimycin A (tyrosine kinase inhibitor), thrombin inhibits proliferation of astrocytes. These effects were accompanied by a decrease in protein tyrosine phosphorylation (Grabham, and Cunningham, 1995). A previous study showed that TRAP and thrombin are not fully agonist for

activation and signal transduction in human platelets (Lau, Pumiglia, et al, 1994). However, both have mitogenic activity, and also can stimulate wound healing. Thus, in an injury situation, thrombin addition would trigger protein tyrosine phosphorylation and stimulate cell proliferation in response to injury (Grabham, and Cunningham, 1995). Additionally, in platelets, TRAP and thrombin induce aggregation, tyrosine phosphorylation, and the formation of phosphatidyl inositol (PtdOH).

Thrombin but not SFLLRNP causes translocation of p44/42 MAPK from the cytosol to the nucleus. Also thrombin, but not TRAP produces large and irreversible platelet aggregation. For the biochemical signaling, thrombin can stimulate longer lasting and greater of the signal than TRAP. In contrast, TRAP causes a transient response due to the metabolism of the peptide (Lau, Pumiglia, et al, 1994; McLaughlin, Shen, et al, 2005).

PAR-1 is the agonist peptide for thrombin that release VEGF in culture medium through PAR-1 activation. Thrombin and SFLLRNP cause release of VEGF in vascular smooth muscle cells. By treating human aortic vascular smooth muscle (HVSM) cells with 10 U/ml of thrombin, PI3K/AKT at Ser473 and Thr308 were activated in the cells. In addition, by using MEK1/2 inhibitor: U0126, PI3K inhibitor: LY294002, and PKC inhibitor: calphostin C, it was shown that these inhibitors, as well as PKC and p44/42 are involved in thrombin induced VEGF release in HVSM cells (Arisato, Sarker, et al, 2003). These results suggest, that

thrombin may have a crucial role in vascular remodeling, by activating the Ras/Raf/MEK/ERK and PI3K/AKT pathways.

1.3.3. Thrombin inhibitors (Serpin)

It was shown the balance between serine proteases and serpins have a role in neuronal system development. Serpins are a group of proteins with similar structures that were first identified as a set of proteins able to inhibit proteases. The name serpin is derived from its activity: as serine protease inhibitor.

Thrombin caused inhibition of morphological differentiation, but by using protease inhibitors, such as protease nexin I (PN-1), stimulated neurite extension. In cultured murine neuroblastoma neurons, thrombin was found to inhibit neurite extension, but by replacing the thrombin containing serum-free media with media lacking thrombin neurite extension occurred. In neuroblastoma and chick sympathetic cells, adding glia-derived nexin (GDN), a serine protease inhibitor, the neurite outgrowth was promoted. Also, in neuroblastoma cells, the protease inhibitors leupeptin and hirudin promote neurite extension, (Gurwitz, and Cunningham, 1988). The detrimental effect of high concentrations of thrombin to cells, that can cause cell death, also can be prevented by using protease inhibitors. In addition, in neuroblastoma cells, thrombin induced cell rounding can be prevented by broad-specificity kinase and tyrosine phosphatase inhibitors

(Jalink, and Moolenaar, 1992), indicating the important involvement of kinases and phosphatases in thrombin-mediated effects on neurites.

On the other hand, one of the protein kinase inhibitors, staurosporin or H-7, inhibit thrombin and prevent neurite retraction. This retraction prompts a feedback loop in which protein kinases are activated to prevent prolonged thrombin-related effects (Suidan, Stone, et al, 1992). This suggests the imbalance between serine protease and their inhibitor could play a role in Alzheimer's disease (Farmer, Sommer, and Monard, 1990; Monard, Niday, et al, 1983). Also, thrombin degrades Tau protein, and in its failure causes Tau aggregation, thus it involved in neurodegenerative disease (Arai, Miklossy, et al, 2006). By immunoreactivity, thrombin was found in senile plaques and some diffuse amyloid deposits and in intracellular neurofibrillary tangles in the brain of people with Alzheimer disease (Arai, Miklossy, et al, 2006).

Additionally, it was found that after brain injury PN-1 was upregulated by this counteracting thrombin. This clearly contributes to the repair process; in case if thrombin is not regulated by any of its inhibitors, eventually causes cell death by caspase activation (Turgeon, Salman, and Houenou, 2000). Western blot analysis showed, the rat brain contains mRNA of glia-derived nexin (GDN) and PN-1 (Cunningham, and Gurwitz, 1989). After nerve injury, thrombin increases, and followed by production of PN-1 that is capable of neutralizing the excessive thrombin which increases twofold: suggest their role in neuronal tissue repair

(Festoff, Smirnova, et al, 1996; Monard, Suidan, and Nitsch, 1992; Smirnova, Ma, et al, 1996). The balance between thrombin and serpins appears to mediate the development and plasticity of the nervous system.

1.3.4. Role of thrombin in the nervous system

Normal development of the nervous system is dependent on neuronal pruning; certain neurons are programmed to die. These neurons can not accomplish proper axonal contact, and synaptogenesis does not occur, this also happens to neurons that do not have sufficient access to target-derived neurotrophic factors (Dennis, 1981). The neurotrophic factor receptors are expressed in neuronal and non- neuronal cells such as glia, and the activation of PARs on these cells leads to cell de-differentiation and cell death. PAR signaling is dependent on the external stimuli and can mediate both cell survival and cell death, depending on the stimuli. In brain tissue, PARs ability to mediate both neurodegeneration and neuroprotection, makes it a strong candidate for a new therapeutic target in neurodegenerative disorders (Luo, Wang, and Reiser, 2007).

Moreover, thrombin was found to induce phosphorylation of p38 MAPK, and use of a p38 MAPK inhibitor, SB203580, inhibits the proliferative effect of thrombin (Kanda, Nishio, et al, 2001). Thus, it appears that thrombin-induced proliferation in smooth muscle cells occurs via the same pathway as that utilized by NGF to induce neurite extension in neuronal cells. Thrombin's inhibition of neurite

outgrowth in DRG is caused by the activation of a G protein-coupled cell surface PAR by cleaving between Arg42 and Ser43 of PAR's extracellular domain. This exposes a new N-terminus which acts as a tethered ligand that initiates signal transduction pathways.

It has been proposed that thrombin initiates smooth muscle cell proliferation by PAR-1 receptor activation, also causes activation of $p21^{ras}$, then binds to GTP and activates Raf-1. Raf-1 is then translocated to the plasma membrane where it phosphorylates MEK1/2. And this in turn activates p44/42 MAPK, and subsequently activates cyclin D1 and transcription factors such as c-Fos to cause increased cell proliferation (Lee, Johnson, et al, 2001). In a separate study using human tracheal smooth muscle cells, the proliferation correlated with ERK phosphorylation in a time and concentration dependent manner. Tyrosine kinase and PI3-K inhibitors attenuated these responses, whereas MEK1/2 inhibitors and overexpression of dominant negative Ras and Raf mutants suppressed the activation of MAPK (Lin, Shyr, et al, 2001; Spencer, Shao, and Andres, 2002), further indicating the importance of the Ras/Raf/MEK/MAPK pathway in thrombin-mediated cell proliferation according to the DNA synthesis in vascular smooth muscle cells. On the other hand, in the cerebral cortex and striatum, thrombin causes a cytotoxic effect through the MAPK pathway. By inhibiting ERK pathway, could reduce thrombin-induced loss of neurons, as reflected by the decrease in the injured area. The same is observed when p38 MAPK is inhibited. This shows that ERK, p38, and JNK are all involved in neuronal death in the

striatum. Thus, cortical injury induced by thrombin can be regulated by the MAPKs pathways (Fujimoto, Katsuki, et al, 2007).

As indicated above, thrombin activity results in neurite retraction, but the mechanism for this process is still largely unknown. Ras and Rho have been shown to be involved in thrombin-induced response. Also it can mediate PI3K and MAPK pathway activation (Wang, and Reiser, 2003). A study in smooth muscle cells illustrated the mitogenic effect of thrombin mediating cell proliferation through the activation of the Ras/Raf/MEK/MAPK pathway. Thus, the MEK1/2 inhibitor, PD98059, and the dominant negative forms of Ras and Raf are useful tools to determine the effect of thrombin on ERK. These experiments showed thrombin causes activation of Ras, Raf and subsequently ERK (Lin, Shyr, et al, 2001). In response to thrombin, MAPK is translocated to the nucleus initiating transcription by interacting with specific transcription factors and affecting cell cycle entry (Turgeon, and Houenou, 1997). Thus, it seems that thrombin can activate signaling pathways that are also activated by NGF. Potentially, thrombin and NGF activate overlapping signaling pathways in neurons.

2. Preliminary Studies

Although there is increasing evidence that thrombin causes retraction of neurites, the underlying mechanisms remain to be investigated. In an attempt to examine the mechanisms leading to thrombin-induced neurite retraction, we initially sought to examine the morphological effects of thrombin in both undifferentiated and differentiated PC12 cells either in the presence or absence of NGF. Consistent with previous findings, neurites of NGF-treated differentiated cells retract following exposure to thrombin. In undifferentiated PC12 cells, thrombin did not cause significant changes in cell morphology in low serum media. However, when undifferentiated PC 12 cells were exposed to NGF and thrombin at the same time, we observed a significant increase in the number and length of neurites in these cells compared to cells treated with NGF alone. It is possible that thrombin amplifies the NGF-induced p38 MAPK/p44/42 MAPK and PI3-K/AKT signaling pathways to enhance NGF-associated neurite extension/neuronal differentiation. On the other hand, thrombin may stimulate distinct signaling pathways in differentiating and differentiated neurons to cause opposing effects on neurites.

3. Hypothesis, Objective & Specific Aims

3.1. Hypothesis

Thrombin stimulates distinct signaling pathways in differentiating and differentiated neurons to cause opposing effects on neurites.

3.2. Objective

To analyze the thrombin-induced signaling pathways leading to neurite retraction and enhanced NGF-mediated neurite extension.

3.3. Specific Aims

The specific aims are:

1. To analyze the signaling pathway through which thrombin induces neurite retraction in differentiated PC12 cells.
2. To examine the signaling pathway through which thrombin enhances NGF-induced neurite extension in PC12 cells.

Chapter 2. Materials and Methods

2.1. Materials

NGF and thrombin were purchased from Sigma. Horse serum, fetal bovine serum (FBS), and RPMI 1640 with L-glutamine were purchased from Invitrogen. Kinase inhibitors (U0126, LY294002 and SB203580) were purchased from Calbiochem. Antibodies for p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), p44/p42 MAPK, phospho-p44/p42 MAPK (Thr202/Tyr204), AKT, and phospho-AKT (Ser473) were purchased from Cell Signaling. Actin (I-19) antibody was purchased from Santa Cruz Biotech. β-Tubulin III and Cy3-conjugated sheep anti-mouse IgG were purchased from Sigma. Donkey anti-goat IgG-HRP and goat anti-rabbit IgG-HRP were purchased from Cell Signaling.

2.2. Cell culture

PC12 cells were cultured in 10-cm dishes in proliferating media (PM: RPMI 1640 with L-glutamine, 5% fetal bovine serum, 10% horse serum, and 100 units penicillin/100 μg streptomycin) at 37°C with 5% CO_2. Cells were passaged three times per week using 0.25% trypsin-EDTA solution (Invitrogen).

2.3. Cell lysis

Cells were lysed in 40 μl of boiling 1X sample buffer (62.5 mM Tris-HCl pH 6.8, 12.5% glycerol, 1.25% SDS, and 1.25% β-mercaptoethanol). Prior to SDS-

PAGE analysis, lysates were centrifuged at 15,000 rpm for 30 sec, sonicated for 10 min, and boiled for 5 min at 95°C.

2.4. Protein assay

Protein concentration of the samples were determined by Bradford microassay as suggested by the manufacturer (Bio-Rad). Bovine serum albumin (BSA) was used as a standard.

2.5. SDS-PAGE and Western blot analysis

Cell lysates (protein amounts normalized by Bradford microassay) were subjected to SDS-PAGE (12.5%) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) in transfer buffer (25 mM Tris base, 200 mM glycine, and 20% (v/v) methanol, pH=8.3). After blocking in 5% non-fat milk in Tris-buffered saline with Tween-20 (TBS/T: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.6 mM KCl, 0.1% Tween-20) for 1 hr at room temperature, membranes were probed with the appropriate primary antibody. After 4 washes with TBS/T, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2500; incubated for 1 hr at room temperature). The ECL Western blotting detection reagents (GE Healthcare) were used to detect the presence of immunoreactive bands. In all experiments, TBS/T was used for antibody dilution.

2.6. Stripping and reblotting

Membranes were incubated in stripping buffer (62.5 mM Tris-HCl, pH=6.7, containing 0.1M β-mercaptoethanol and 2% SDS) and incubated at 55°C for 30 min with shaking. After washing with TBS/T 6 times (five min each wash), membranes were subjected to Western blot analysis as described above.

2.7. Immunocytochemistry

Cells seeded on poly-L-lysine coated coverslips were gently washed in 1X PBS, fixed in cold (-20°C) absolute methanol for 10 minutes at 4°C and kept in 1X PBS at 4°C until the day of staining. Fixed cells were probed with β-tubulin III antibody (diluted to 1:150) followed by incubation with Cy3-conjugated goat anti-mouse IgG (diluted to 1:200). Both primary and secondary antibody incubation times were 40 min at room temperature. Cell nuclei were stained with DAPI (diluted to 1:150) for five minutes at room temperature. Permafluor aqueous mounting medium (Beckman) was used to mount coverslips onto slides.

2.9. Differentiation and neurite retraction assays and time-lapse microscopy

PC12 cells plated in PM at a density of 1.5 x 10^5 cells per 35-mm plate for 24 hrs were switched to differentiating media (DM; RPMI 1640 with L-glutamine, 0.2% horse serum, and 100 unit penicillin/100 μg streptomycin). After serum starvation (in DM) for 18 hours, cells were treated with NGF (100 ng/ml) to induce differentiation. Differentiated PC12 cells were then photographed 48 hours after treatment using an Olympus IX71 inverted microscope. For neurite retraction assay, differentiated PC12 cells were exposed to thrombin (1 unit/ml) and retraction of neurites was monitored/photographed every minute for 1 hour using an Olympus 1X17 inverted microscope. Live cell images and length of neurites of cells treated with NGF or NGF+thrombin were analyzed using the Image-Pro software (Olympus).

2.10. Statistical analysis

All statistical analysis was done using one-way ANOVA in SPSS 13.0. Data was considered significant only when the p-value was smaller than 0.05.

Chapter 3. Results

3.1. Thrombin induces neurite retraction in differentiated cells via activation of AKT but not p44/42 and p38 MAPK.

Thrombin is known to stimulate neurite retraction but the molecular mechanisms remain to be investigated. My goal was to analyze the signaling pathway through which thrombin induces neurite retraction in differentiated PC12 cells, a model system commonly used for neuronal studies. PC12 cells cultured in low serum media for 18 hrs were treated with NGF (100 ng/ml) for 48 hours. To examine retraction of neurites in differentiated PC12 cells, time-lapse microscopy using an Olympus 1X17 microscope was performed after addition of thrombin (1 unit/ml) into the culture media. As shown in Fig. 1, NGF-differentiated PC12 cells (time 0) generally exhibited long neurites after 72 hrs. By 28 min, neurites from some of the cells have clearly retracted, and by 60 min, most of the neurites (95%) disappeared. Indeed, thrombin causes a dramatic retraction of neurites in differentiated PC12 cells. Having successfully induced retraction of neurites by thrombin treatment, we proceeded to analyze the signaling pathway involved in the process. To do so, cells were plated in PM at a density of 1.5×10^5 cells in a 35 mm plate for 24 hrs then switched to low serum DM. After serum starvation for 18 hours, cells were induced to differentiate by NGF treatment. After 48 hrs, the cells were exposed to thrombin at different time points and activation of p38 MAPK, p44/42 MAPK (ERK1/2) and AKT were evaluated. These kinases were chosen to be analyzed as they have been previously implicated in a number of signaling mechanisms,

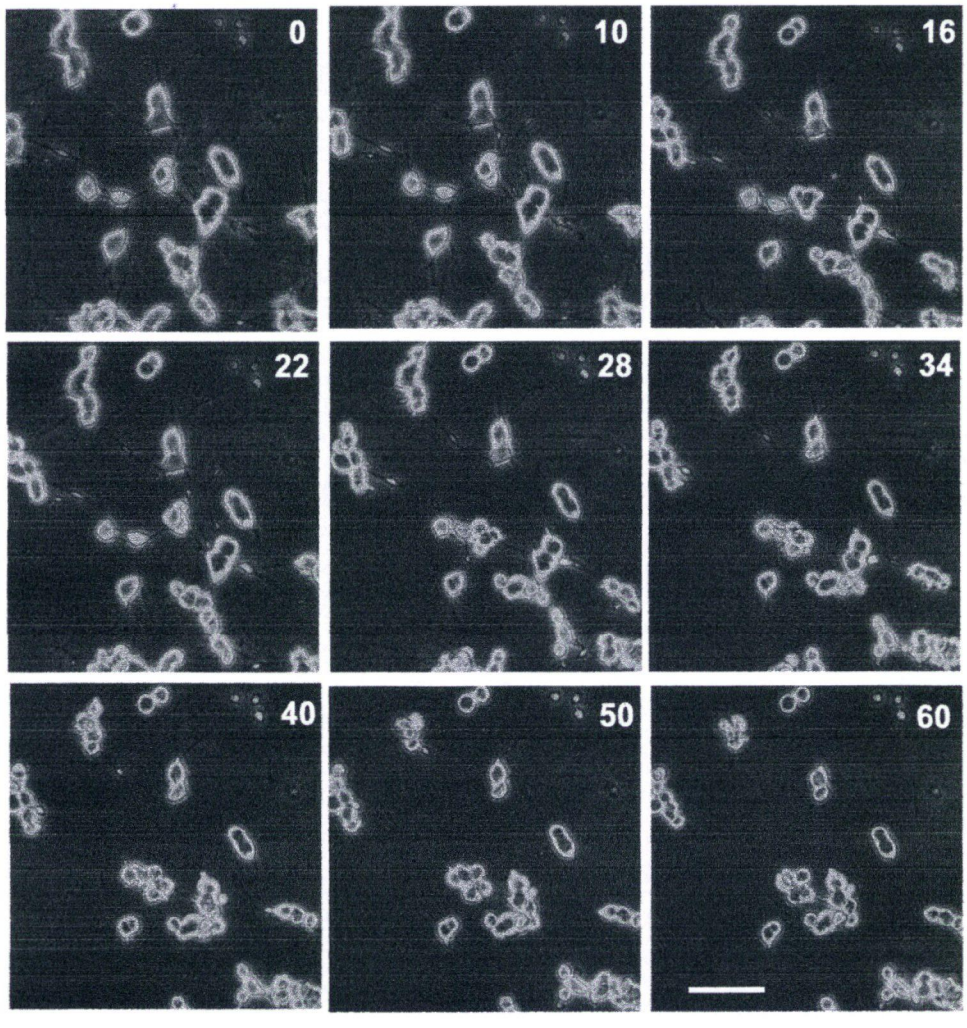


Fig. 1.

Fig. 1. Thrombin induces dramatic neurite retraction in differentiated cells. PC12 cells plated in proliferating media at a density of 1.5 x 10^5 cells in a 35 mm dish for 24 hrs were switched to low serum DM. After serum starvation for 18 hours, cells were treated with NGF (100 ng/ml) for 72 hours to induce differentiation. After that, thrombin (I unit/ml) was added to induce retraction of neurites from differentiated cells. To follow neurite retraction, time-lapse microscopy/ photographs were taken every minute for 60 min using an Olympus 1X17 microscope. The numbers on the top right corner of every panel indicate the time (min) after addition of thrombin. Scale bar=100 μM

including cell differentiation, survival and neurite retraction. By Western blot analysis using phospho-specific antibodies, we found that p44/42 MAPK and p38 MAPK had only modest changes in activation following thrombin treatment (Fig. 2A and Fig. 2B, top panels). However, we found that AKT was clearly activated 3 min (Fig. 2A, lane 2; Fig. 2B bottom panel) after thrombin treatment. AKT activation was sustained and reached a peak at 10 min (Fig. 2A, lane 3, Fig. 2B bottom panel) and declined at 30 min (Fig. 2A, lane 4; Fig. 2B bottom panel) following thrombin treatment. No apparent AKT activation was detected 60 min (Fig. 2A, lane 5; Fig. 2B bottom panel) after exposure to thrombin. Thus, it appears that thrombin induces neurite retraction in differentiated PC12 cells through the activation of AKT but not p44/42 and p38 MAPK.

To further investigate the involvement of AKT in thrombin-mediated neurite retraction, we examined the effects of LY294002, an inhibitor of the AKT upstream kinase, PI3K, in differentiated PC12 cells. For this study, differentiated cells were incubated with 30 μM LY294002 for 30 minutes and subsequently treated with thrombin. To examine for neurite retraction following thrombin treatment, time-lapse microscopy/photographs were taken every minute for 60 min. As shown in Fig. 3, only a minimal change in neurites was noted 60 min after thrombin treatment, indicating that pre-treatment with LY294002 inhibits thrombin-induced neurite retraction in differentiated PC12 cells. This result further supports our earlier finding that activation of AKT is involved in thrombin-induced neurite retraction in differentiated PC12 cells.

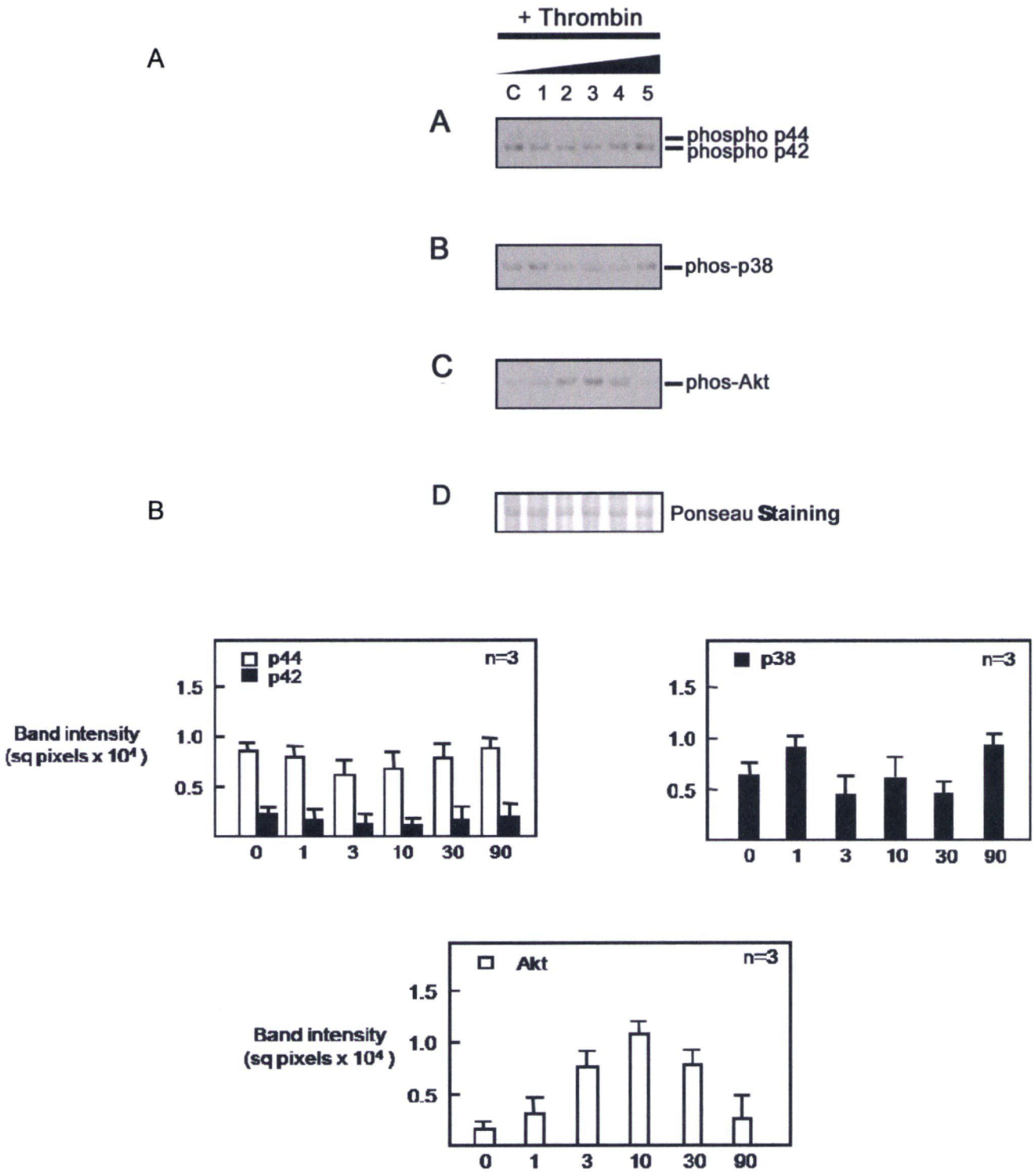

A
+ Thrombin
C 1 2 3 4 5
A
phospho p44
phospho p42
B
phos-p38
C
phos-Akt
B
D
Ponseau Staining
p44
p42
n=3
Band intensity
(sq pixels x 10⁴)
1.5
1.0
0.5
0 1 3 10 30 90
p38
n=3
1.5
1.0
0.5
0 1 3 10 30 90
Akt
n=3
Band intensity
(sq pixels x 10⁴)
1.5
1.0
0.5
0 1 3 10 30 90

Fig. 2

Fig. 2. AKT is activated during thrombin-induced neurite retraction in differentiated cells. A. Differentiated PC12 cells were treated with thrombin (1 unit/ml) to induce neurite retraction. Cells were lysed at various time points (0, 1, 3, 10, 30, 60 min; lanes C, 1, 2, 3, 4, 5, respectively) following thrombin treatment and lysates were subjected to Western blot analysis using phospho-antibodies to p44/42 MAPK, p38 MAPK, and AKT. B. Intensities (in square pixels) of the immunoreactive bands in (A) were determined by densitometric analysis using the NIH Image 1.61. software. Values are means±SD of three separate experiments.

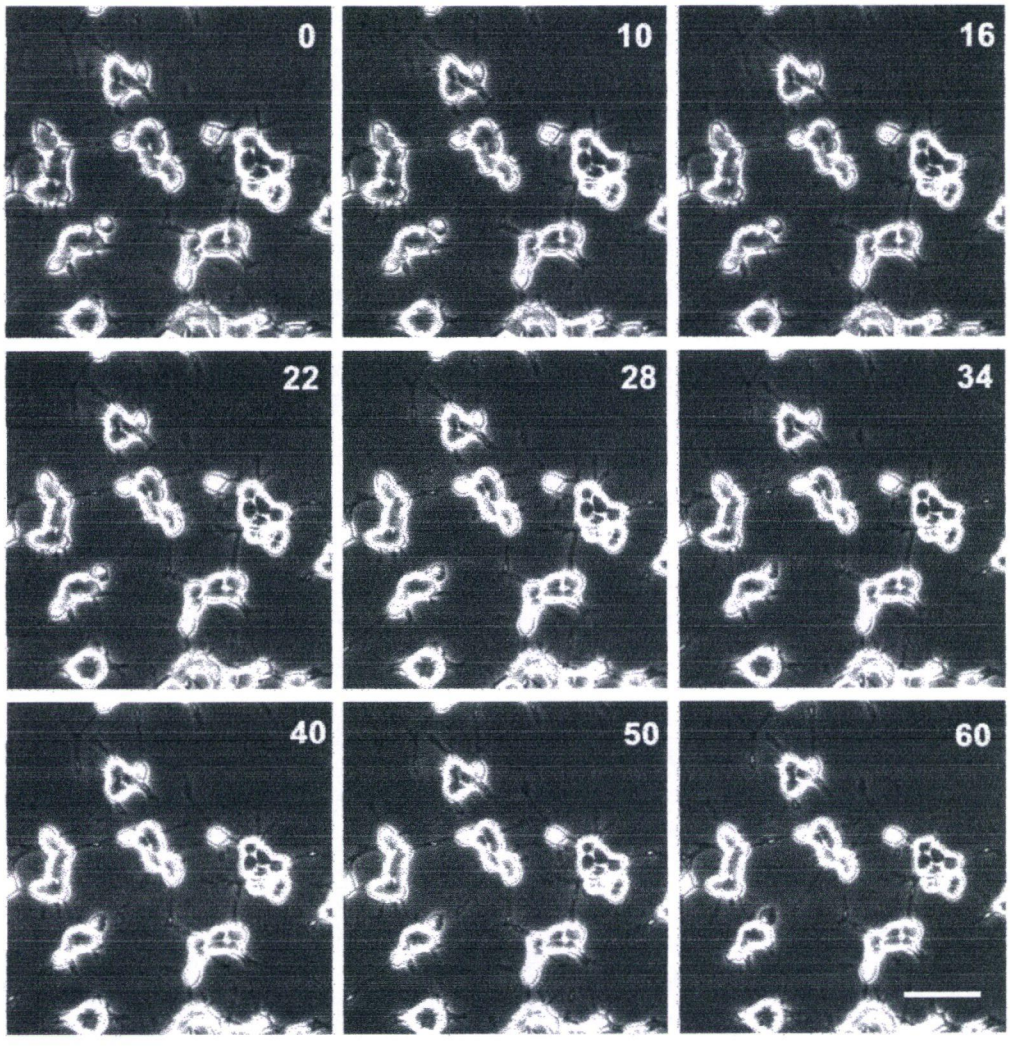


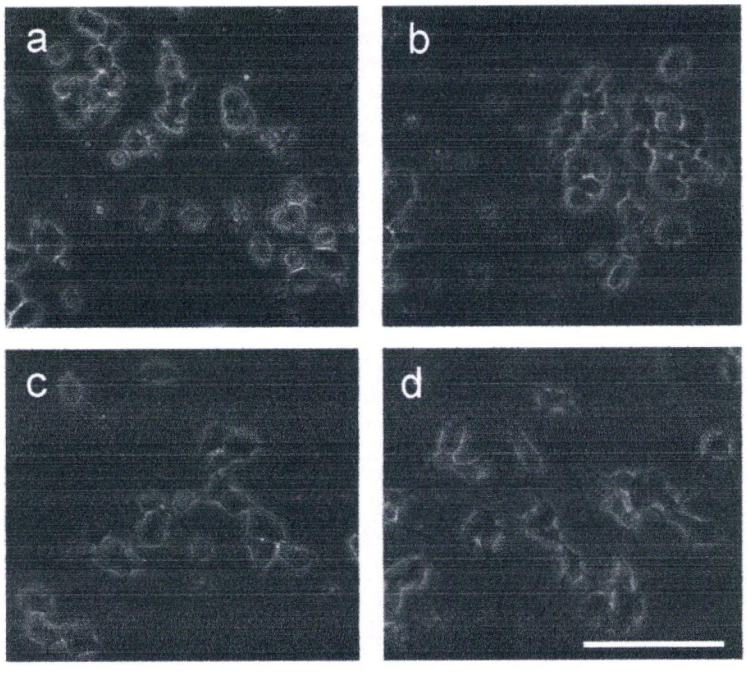
Fig. 3

Fig. 3. LY294002 inhibits thrombin-induced neurite retraction in differentiated PC12 cells. PC12 cells plated in PM at a density of 1.5 x 10^5 cells in a 35 mm plate for 24 hrs were switched to DM. After serum starvation for 18 hrs, cells were induced to differentiate by NGF treatment. After 72 hours, cells were pre-incubated with LY294002 (30 μM) for 30 minutes at 37°C then treated with thrombin. Changes in neurites of differentiated PC12 cells were monitored by taking live cell images every minute for 60 min using an Olympus 1X17 microscope. The numbers on the top right corner of every panel indicate the time (min) after addition of thrombin. Scale bar=100 μM

3.2. Thrombin synergistically enhances NGF-induced neurite outgrowth in differentiating cells.

Given that thrombin retracts NGF-induced neurites in differentiated cells, I sought to examine whether thrombin would inhibit growth of neurites in cells induced to differentiate by NGF. To do so, serum starved, undifferentiated PC12 cells were treated with thrombin alone, NGF alone, or NGF and thrombin at the same time. The cells were then photographed 48 hours after treatment. As shown in Fig. 4A, thrombin did not cause a significant change in the morphology of undifferentiated cells in low serum media (panel b). As expected, exposure of undifferentiated cells to NGF induced neurite outgrowth (panel c). Surprisingly, however, simultaneous addition of NGF and thrombin resulted in longer neurites (panel d). A comparison of cells treated with NGF alone and NGF+thrombin (Fig. 4B) revealed a significant increase in length of neurites (left panel) and number of cells with long (≥ 20 μm) neurites (right panel) in NGF+thrombin-treated cells. These results indicate that thrombin synergistically enhances NGF-induced neurite outgrowth in differentiating PC12 cells, which clearly express neuronal β-tubulin III (Fig. 4C). Positive staining in control and thrombin-treated cells may be due to endogenous production of NGF in PC12 cells under low serum DM.

A


a
b
c
d

B

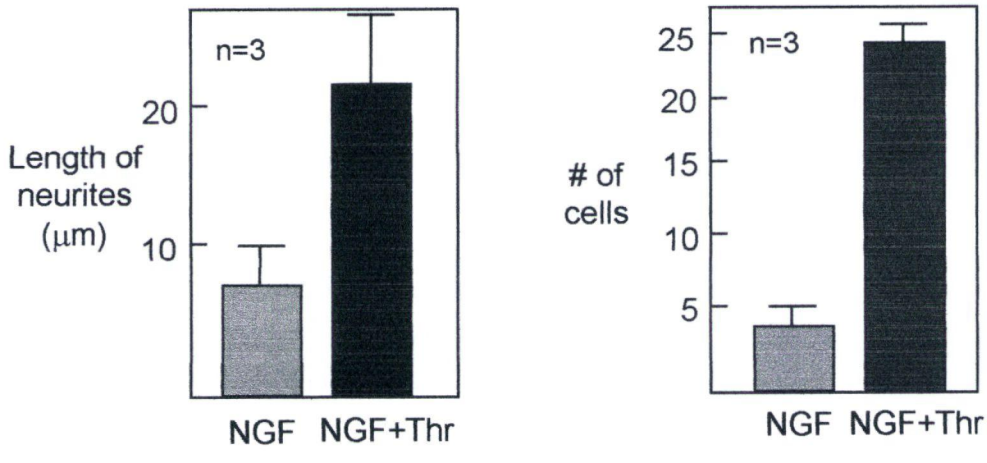

n=3
Length of neurites (μm)
20
10
NGF
NGF+Thr
n=3
# of cells
25
20
15
10
5
NGF
NGF+Thr

Fig. 4

C

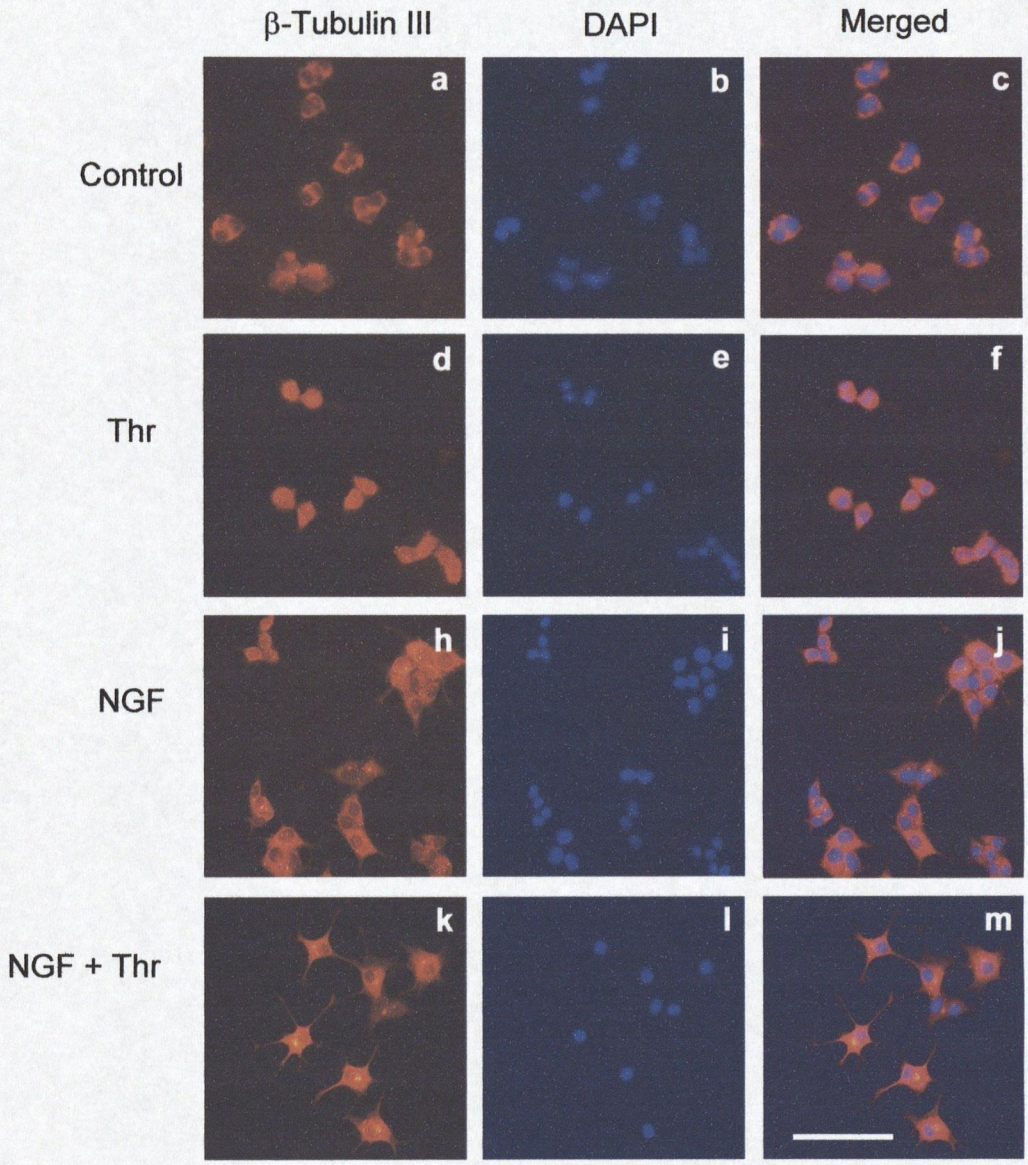

β-Tubulin III
DAPI
Merged
Control
Thr
NGF
NGF + Thr
a
b
c
d
e
f
h
i
j
k
l
m

Fig. 4

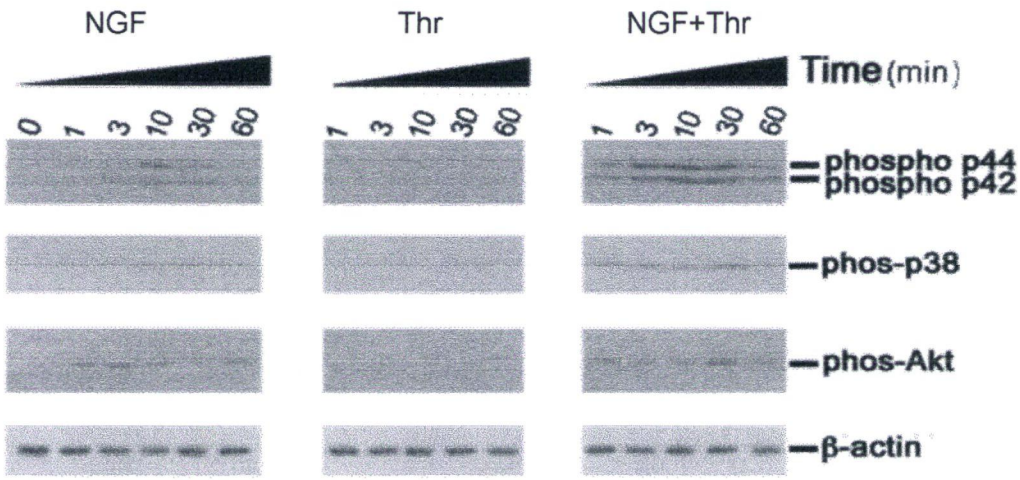
Fig. 4. Thrombin synergistically enhances NGF-induced neurite outgrowth in differentiating cells. A. Cells plated in PM at a density of 1.5×10^5 cells in a 35 mm plate for 24 hrs were switched to DM. After 18 hours in low serum DM, cells were treated with thrombin alone (panel b), NGF alone (panel c), or NGF and thrombin at the same time (panel d). In panel a, cells were not treated with either thrombin or NGF. The cells were photographed 48 hours after treatment. B. The left panel compares the length of neurites of cells treated with NGF and NGF+thrombin. Measurements were taken from 150 cells in each treatment (n=3). The right panel shows the number of cells (out of 150 cells for each treatment) with neurites longer than 20 μm (n=3). The significant difference, using one-way ANOVA, $p \leq 0.001$, $p \leq 0.01$) respectively. C. Serum-starved control cells (a,b,c), cells treated with thrombin alone (d,e,f), NGF alone (h,i,j), and NGF+thrombin (k,l,m) were stained with β-tubulin III (left panels) and DAPI (middle panels). The right panels are merged images of the left and middle panels. Each scale bar=100 μM

3.3. Thrombin augments NGF-induced neurite outgrowth in differentiating cells via enhanced and sustained activation of p44/42 and p38 MAPK.

The opposing effects of thrombin in differentiated and differentiating PC12 cell neurites suggest stimulation of distinct signaling pathways in these cells. Knowing that AKT is involved in thrombin retraction of NGF-induced neurites in differentiated cells, we now sought to analyze the signaling pathway through which thrombin enhances NGF-induced neurite outgrowth in differentiating cells. For this study, cells in low serum DM treated with NGF alone, thrombin alone, or NGF and thrombin at the same time, were lysed in 1X SDS-PAGE sample buffer at different time points following treatment. Protein samples were then resolved in 12.5% SDS-PAGE and subjected to Western blot analysis using phospho-antibodies against p44/42 MAPK, p38 MAPK and AKT. Consistent with previous reports (Kim, Seger, et al, 2004; Burry, 2001), we found activation of p44/42 MAPK, p38 MAPK and AKT in NGF-stimulated PC12 cells (Fig. 5, A and B, left panels). Treatment with thrombin alone did not cause significant activation of any of these kinases (Fig. 5, A and B, middle panels). However, simultaneous treatment with NGF and thrombin resulted in enhanced and sustained activation of p44/42 MAPK and p38 MAPK (Fig. 5, A and B, right panels). Conversely, activation of AKT was similar to that observed in cells treated with NGF alone.

To further investigate the involvement of p44/42 MAPK and p38 MAPK in the enhancement of NGF-induced neurite extension in differentiating cells, we took

A


NGF
Thr
NGF+Thr
Time (min)
0 1 3 10 30 60
1 3 10 30 60
1 3 10 30 60
phospho p44
phospho p42
phos-p38
phos-Akt
β-actin

B

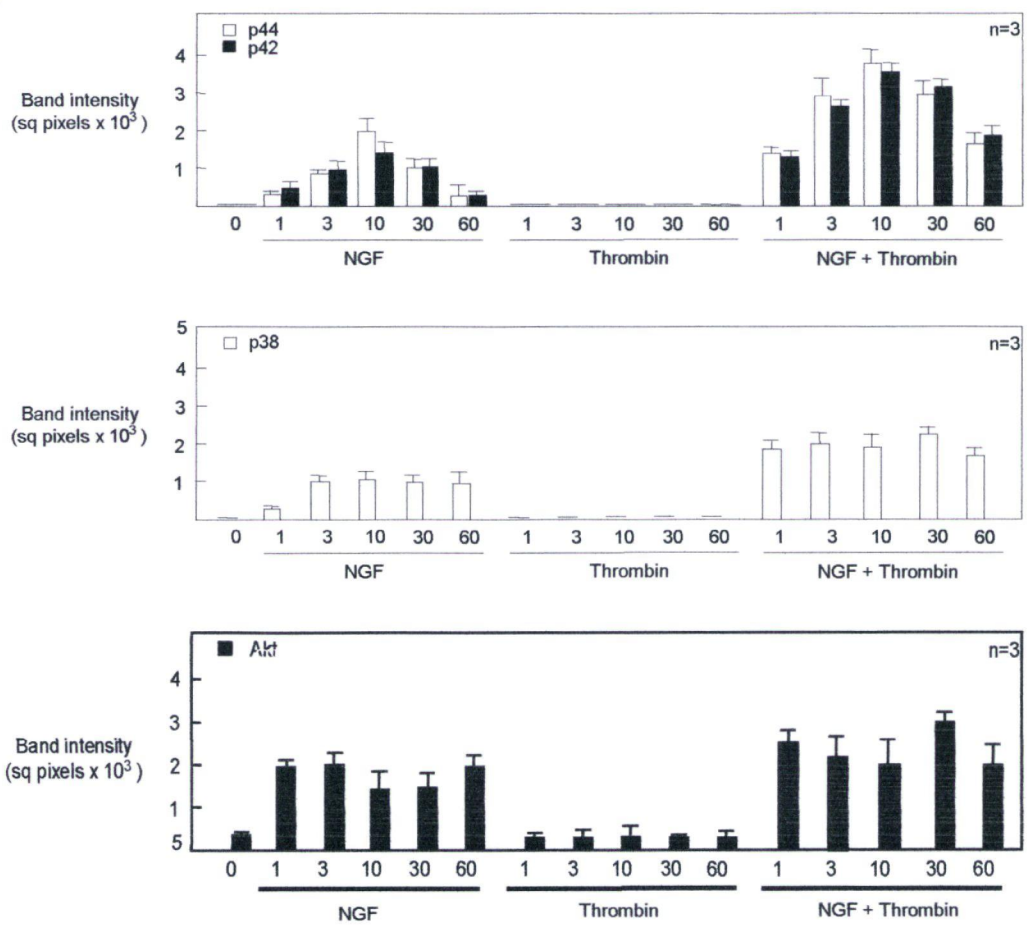

p44
p42
n=3
Band intensity (sq pixels x 10³)
0 1 3 10 30 60 1 3 10 30 60 1 3 10 30 60
NGF
Thrombin
NGF + Thrombin
p38
n=3
Band intensity (sq pixels x 10³)
NGF
Thrombin
NGF + Thrombin
Akt
n=3
Band intensity (sq pixels x 10³)
NGF
Thrombin
NGF + Thrombin

Fig. 5

Fig. 5. Thrombin enhances NGF-induced neurite outgrowth in differentiating cells via enhanced and sustained activation of p44/42 and p38 MAPK. A. PC12 cells plated in PM at a density of 1.5 x 10^5 cells in a 35 mm plate for 24 hrs were switched to DM. After serum starvation in DM for 18 hours, cells were treated with NGF alone (left panels), thrombin alone (middle panels), or NGF and thrombin simultaneously (right panels), and lysed at 0, 1, 3, 10, 30 and 60 minutes post-treatment in boiling 1X sample buffer. Equivalent amounts of protein samples were then resolved in 12.5% SDS-PAGE and subjected to Western blot analysis using phospho-antibodies against p44/42 MAPK, p38 MAPK, AKT. A blot for β-actin was used as loading control. B. Intensities (in square pixels) of the immunoreactive bands in (A) were determined by densitometric analysis using the NIH Image 1.61. software. Values are means±SD of three separate experiments.

advantage of the presence of the p44/42 MAPK and p38 MAPK inhibitors, U0126 and SB203580, respectively. Serum starved PC12 cells were then exposed to SB203580 or U0126 at a concentration of 10 μM for 30 min prior to simultaneous treatment with NGF and thrombin. A set of cells were also pre-exposed to LY294002 to verify that AKT does not play an important role in NGF+thrombin-mediated neurite extension. After 48 hours in DM containing NGF and thrombin, we observed a clear decrease in neurite extension in cells exposed to SB203580 and U0126 (Fig. 6, A and B). Cells exposed to LY294002 did not show a notable decrease in neurite extension. In these studies, we determined by Western blot analysis using phospho-specific antibodies that we have effectively inhibited p44/42 MAPK, p38 MAPK and AKT by using U0126, SB203580, and LY294002, respectively. Thus, these data support the notion that thrombin+NGF-induced neurite extension is achieved via enhanced and sustained activation of p44/42 MAPK and p38 MAPK.

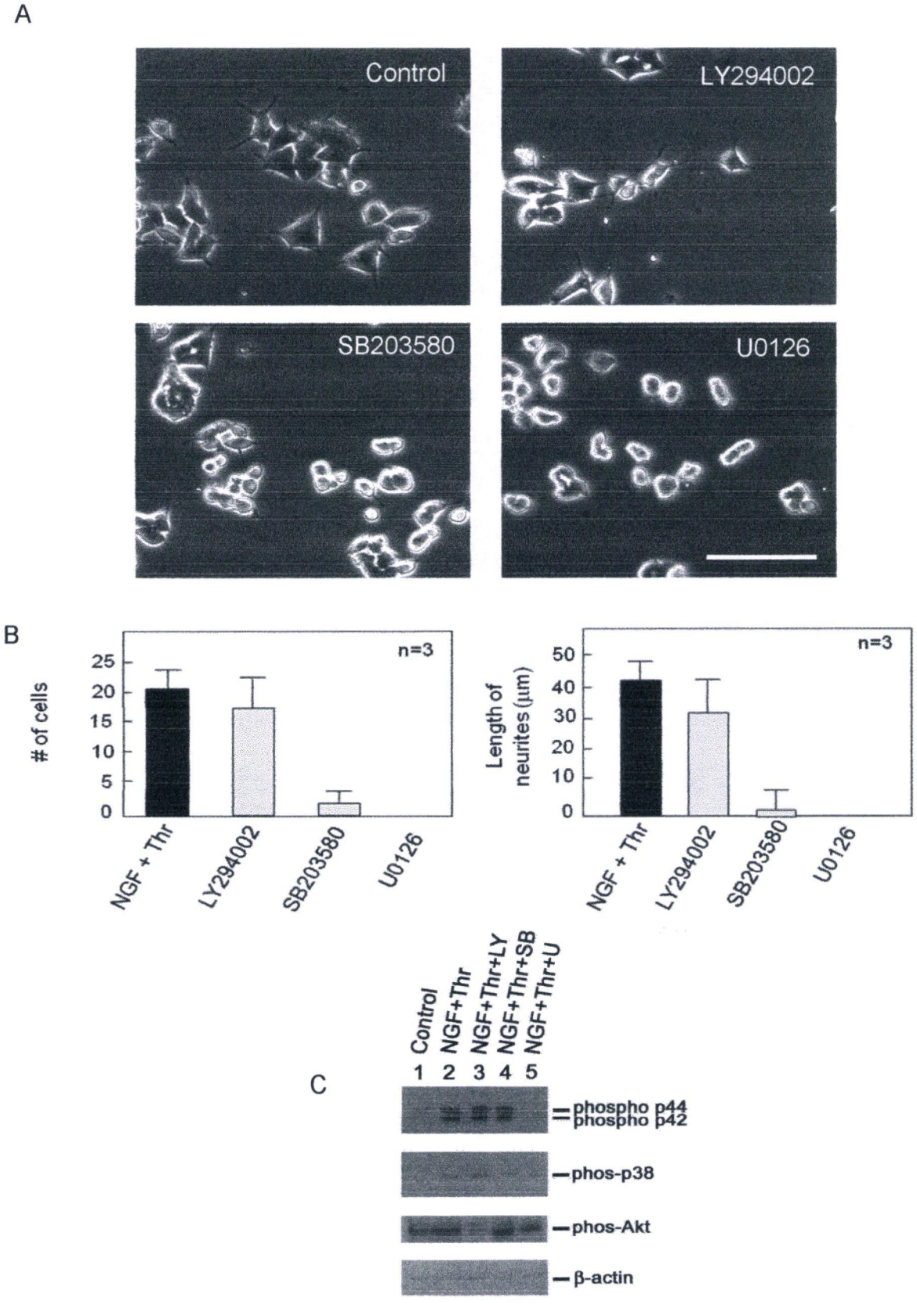

A
Control
LY294002
SB203580
U0126
B
# of cells
n=3
25
20
15
10
5
0
NGF + Thr
LY294002
SB203580
U0126
Length of neurites (μm)
n=3
50
40
30
20
10
0
NGF + Thr
LY294002
SB203580
U0126
C
Control
NGF+Thr
NGF+Thr+LY
NGF+Thr+SB
NGF+Thr+U
1 2 3 4 5
phospho p44
phospho p42
phos-p38
phos-Akt
β-actin

Fig. 6

Fig. 6. Inhibition of p44/42 and p38 MAPK blocks NGF+thrombin-induced neurite extension in differentiating cells. A. Cells plated in PM at a density of 1.5 x 10^5 cells in a 35 mm plate for 24 hrs were switched to low serum DM. After serum starvation for 18 hours, cells were treated with 10 μM of SB203580 (p38 MAPK inhibitor) and U0126 (p44/42 MAPK inhibitor), or 30 μM of LY294002 (inhibitor of the AKT upstream kinase, PI3K) for 30 min prior to treatment with NGF+thrombin. Cells were then photographed 48 hours after culture in NGF and thrombin-containing DM. B. The left panel shows the length of neurites of cells treated with NGF+thrombin in the presence and absence of LY294002, SB203580 or U0126. Measurements were taken from 150 cells in each treatment (n=3). The right panel shows the number of cells (out of 150 cells for each treatment) with neurites longer than 20 μm (n=3). The significant difference, using one-way ANOVA, $p \leq 0.001$, $p \leq 0.01$) respectively. C. Cells grown as indicated above were incubated with LY294002, SB203580 or U0126 for 30 min, and treated with NGF and thrombin for 30 min. Cells lysed in 1X boiling sample buffer were resolved by SDS-PAGE and subjected to Western blot analysis using phospho-antibodies against p44/42 MAPK, p38 MAPK and AKT. A blot for β-actin was used as loading control. Scale bar=100 μM

Chapter 4. Discussion

4.1. Discussion

Neurite remodeling is essential for nervous system development and plasticity. It involves both neurite extension and retraction. Neurite extension is regulated by NGF through phosphorylation and subsequent activation of p44/42, p38 MAPK, and PI3-K/AKT signaling pathways (Fig. 7). NGF binding to TrkA results in endocytosis together with $p75^{NTR}$ and activation of p44/p42 MAPK (Perrone, Paladino, et al, 2005). Previous studies have shown that SB203580, a specific inhibitor of p38 MAPK, causes significant inhibition of neurite outgrowth, indicating that p38 MAPK is also required for neuronal differentiation (Morooka, and Nishida, 1998). Furthermore, the use of p44/42 and p38 MAPK inhibitors in NGF-differentiated cells causes the suppression of the transcription factors, AP-1, c-jun and c-fos, indicating their importance in neuronal differentiation as well (Eriksson, Taskinen, and Leppa, 2007). Neurite retraction, on the other hand, has been shown to be regulated by the serine protease, thrombin. However, the signaling mechanism leading to thrombin-induced neurite retraction has not been previously evaluated. We, therefore, sought to analyze the signaling pathway leading to thrombin-induced neurite retraction in differentiated PC12 cells, a commonly used model system for neuronal studies. Using time-lapse microscopy data and immunoblot analysis, we found that thrombin, indeed, induces neurite retraction in NGF-differentiated PC12 cells. This finding seems to be in harmony with a previous report showing that thrombin can suppress neurite outgrowth in response to stress such as serum starvation (Pai, and

Cunningham, 2002). In my study, thrombin-induced retraction of neurites in NGF-differentiated cells is associated with activation of AKT but not p44/42 MAPK and p38 MAPK.

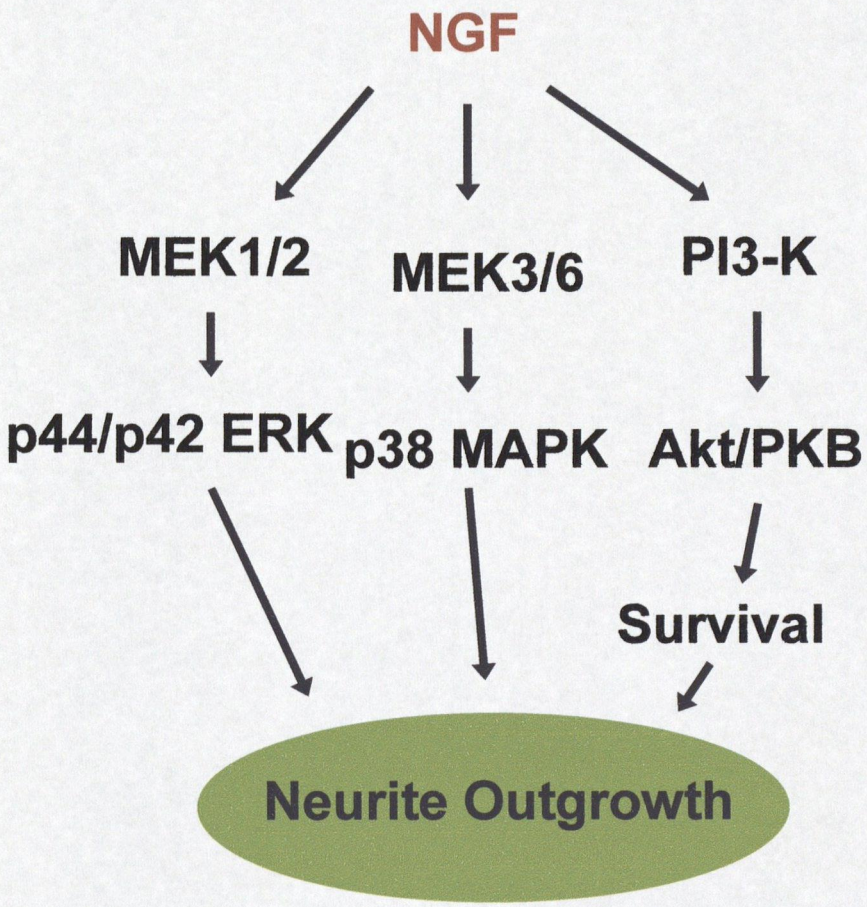


Fig. 7

Fig. 7. **NGF causes neurite extension through activation of p44/42 MAPK and p38 MAPK and PI3-K/AKT signaling pathways.** p44/42 and p38 MAPK are activated by NGF in differentiating cells. The PI3K/AKT survival pathway is also activated by NGF in differentiating cells.

The fact that pre-treatment of cells with LY294002 (an inhibitor of the AKT upstream kinase, PI3K) inhibits thrombin-induced neurite retraction in differentiated PC12 cells support our novel showing that activation of AKT is, indeed, involved in thrombin-induced neurite retraction.

Since thrombin retracts NGF-induced neurites in differentiated cells, our investigations led us to examine whether thrombin would inhibit growth of neurites in cells induced to differentiate using NGF. Our results were unexpected but interesting. I found that simultaneous addition of NGF and thrombin results in a dramatic increase in length of neurites and number of cells with long ($\geq$ 20 μm) neurites compared to cells treated with NGF alone. Thus, as opposed to the neurite retraction effect of thrombin in NGF-differentiated cells, thrombin enhances neurite extension in cells that are differentiating in response to NGF. The latter indicates a novel synergistic effect of NGF and thrombin as well as a novel mechanism for neurite extension that, to our knowledge, have not been previously addressed.

As indicated above, studies in the past have demonstrated that NGF promotes differentiation and neurite extension via activation of p44/p42 MAPK and p38 MAPK (Morooka, and Nishida, 1998; Grewal, York, and Stork, 1999; Yaka, Gamliel, et al, 1998, Rueda, Navarro, et al, 2002). Interestingly, these kinases have also been found to be activated during thrombin-induced proliferation of smooth muscle cells (Lee, Johnson, et al, 2001; Lin, Shyr, et al, 2001, Kanda,

Nishio, et al, 2001). Phosphorylation of p44/p42 MAPK has been linked to subsequent activation of transcription factors (Lin, Shyr, et al, 2001). Interestingly, inhibition of the AKT upstream kinase, PI3K, was shown to attenuate this activation. In the current study, analysis of the signaling pathway that is utilized by thrombin in enhancing NGF-induced neurite extension in differentiating cells revealed enhanced and sustained activation of p44/42 and p38 MAPK. Note that these kinases are apparently not involved in thrombin-mediated signaling in differentiated cells. Although the PI3K/AKT signaling pathway is activated in response to thrombin in differentiated cells, AKT activity in differentiating cells treated simultaneously with NGF and thrombin is similar to that observed in differentiating cells exposed to NGF alone. This supports the view that thrombin stimulates separate signaling pathways in differentiated and differentiating cells.

The amplified and sustained activation of p44/42 and p38 MAPK in differentiating cells exposed to both thrombin and NGF is consistent with the ability of thrombin to induce p44/42 and p38 MAPK in proliferating smooth muscle cells (Lin, Shyr, et al, 2001 ; Fig. 8). While enhanced and sustained activation of p44/42 has also been associated with apoptosis in primary cortical neurons (Ya-Li Zheng et al., MBC 18, 404-413; Fig. 8), we did not observe significant PC12 cell death following simultaneous treatment with NGF and thrombin. This may be explained by the fact that NGF also exerts a neuroprotective effect on PC12 cells by activating the PI3-K/AKT pathway, which is important for cell survival (Liot,

Gabriel, et al, 2004). Indeed, Akt has been found to be responsible for approximately 80% of survival in NGF-treated cells (Kaplan, and Miller, 2000). Thus, we also noted sustained and similar state of AKT activation in cells treated with NGF alone and in cells treated with NGF+thrombin. Together with our finding that inhibition of PI3K, an AKT upstream kinase, does not alter the effect of NGF+thrombin suggest that the PI3K/AKT pathway is not involved in the thrombin enhancement of NGF-induced neurite extension in differentiating cells.

Although thrombin alone does not elicit activation of p44/p42 MAPK, p38 MAPK and AKT in cells that are in NGF-free low serum media, we cannot rule out the possibility that thrombin by itself stimulates other cellular events under this condition. However, these events do not appear to include regulation of neurites and activation of p44/p42 MAPK, p38 MAPK and AKT. Nonetheless, our studies indicate that p44/42 and p38 MAPK activation are important for the synergistic effect of NGF and thrombin on neurite extension in differentiating cells.

In summary, our studies provide clear indications that thrombin elicits opposing effects in differentiated and differentiating neurons via activation of distinct signaling pathways. More importantly, our findings that thrombin and NGF can act in concert to enhance neurite extension through the p44/42 MAPK and p38 MAPK signaling pathways are novel contributions to the fields of brain and signaling research, and have potential clinical applications. Our findings have important implications for example in understanding the development and

diseases of the nervous system, including impaired learning and memory, and spinal cord injury.

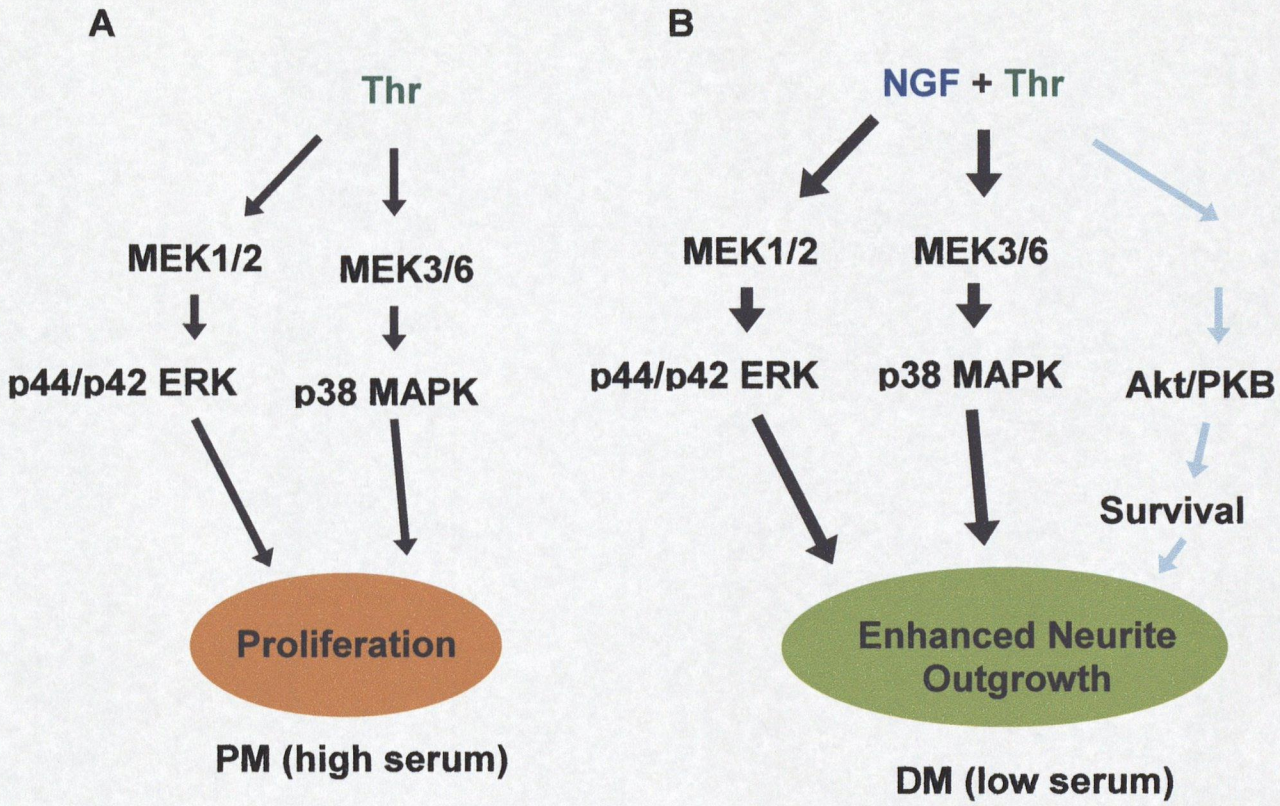

A
Thr
MEK1/2
MEK3/6
p44/p42 ERK
p38 MAPK
Proliferation
PM (high serum)
B
NGF + Thr
MEK1/2
MEK3/6
p44/p42 ERK
p38 MAPK
Akt/PKB
Survival
Enhanced Neurite Outgrowth
DM (low serum)

Fig. 8. Simultaneous addition of NGF and thrombin results in enhanced neurite extension through amplified and sustained activation of p44/42 and p38 MAPK in differentiating cells. A. Thrombin induces proliferation of smooth muscle cells through activation of p44/42 and p38 MAPK in high serum media. B. Simultaneous addition of NGF and thrombin results in enhanced neurite extension via amplified and sustained activation of p44/42 and p38 MAPK in differentiating cells. Activation of the PI3K/AKT survival pathway by NGF exerts a neuroprotective effect in differentiating cells.

4.2. Future Directions

Future studies that include further characterization of the molecular mechanisms leading to and following AKT activation, and p44/p42 MAPK and p38 MAPK activation in differentiated and differentiating neurons, respectively, in response to thrombin would be the next step. Since increasing Cdk5 activity promotes axonal growth, we will also determine whether Cdk5 is involved in thrombin enhancement of NGF-induced neurite extension, by examining whether Cdk5 activity in cells treated with thrombin+NGF is increased. Cdk5 activity will be measured in Cdk5/p35 immunoprecipitates using histone H1 (9-18) peptide as substrate. We will also check whether siRNA directed at Cdk5 will inhibit further neurite extension in (thrombin+NGF)-treated cells. Alternatively, we will confirm the involvement of p44/p42 MAPK and p38 in thrombin+NGF induced neurite extension by depleting p44/p42 MAPK and p38 expression via specific siRNAs. We will also confirm the role of PI3K/AKT in neurite retraction in thrombin-treated differentiated cells by using the same method.

We will then determine whether thrombin causes TrkA transactivation in the presence of NGF by triggering TrkA-PAR-1 interaction and/or subsequent TrkA phosphorylation. TrkA-PAR-1 interaction in Triton X-100-treated membrane fraction of cells treated with NGF+thrombin will be analyzed by immunoprecipitating the complex using either PAR-1 or TrkA antibody or subsequently immunoblotting with Trk A or PAR-1 antibody, respectively. TrkA

phosphorylation will be determined by immunoblotting. Since thrombin could enhance NGF-induced neurite extension by cleavage of NGF to produce a more biologically active molecule, we will perform an *in vitro* assay whereby NGF will be incubated with thrombin, and generation of proteolytic products from NGF will be assessed by HPLC column chromatography. If we detect NGF proteolytic products, we will examine their ability to promote neurite extension in comparison with intact NGF. To complement these studies, we will determine whether the thrombin receptor agonist peptide, SFLLRN-CONH or TFLLRNKPDK, which also induces neurite retraction, causes enhanced NGF-induced neurite extension via a mechanism similar to that of thrombin. These studies will significantly improve our understanding of how thrombin regulates neuronal cell function. Results from these studies will also pave the way for possibly targeting thrombin in certain diseases of the nervous system.

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