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SIGNALING MECHANISMS REGULATING NEURONAL GROWTH CONE DYNAMICS

by

KARINE TORNIERI

Under the Direction of Dr. Vincent Rehder

ABSTRACT

During the development of the nervous system, neurons migrate to their final location and extend neurites that navigate long distances in the extracellular environment to reach their synaptic targets. The proper functioning of the nervous system depends on correct connectivity, and mistakes in the wiring of the nervous system lead to brain abnormalities and mental illness. Growth cones are motile structures located at the tip of extending neurites that sense and respond to guidance cues encountered along the path toward their targets. Binding of these cues to receptors located on growth cone filopodia and lamellipodia triggers intracellular signaling pathways that regulate growth cone cytoskeletal dynamics. Although studies on extracellular cues and their effects on neuronal guidance are well documented, less is known about the intracellular signaling mechanisms that regulate growth cone motility. This dissertation focuses on two signaling pathways and describes how they might be involved in determining growth cone morphology during neuronal development. The specific aims of this work address: (1) the

role of phosphatidylinositol-3-kinase (PI-3K) and its downstream signaling pathway in regulating growth cone motility, and (2) the effect of nitric oxide (NO) release from a single cell on growth cone morphology of neighboring neurons.

This study employs defined neurons from the pond snail, *Helisoma trivolvis*, to demonstrate that inhibition of PI-3K induces a concomitant increase in filopodial length and a decrease in the rate at which neurites advance. These effects are mediated through the lipid and protein kinase activities of PI-3K, and filopodial elongation is due to an increase in the rate at which filopodia elongate and the time that individual filopodia spend extending. Additionally, this study demonstrates that NO release from a single cell can affect growth cone dynamics on neighboring neurons via soluble guanylyl cyclase (sGC), and that NO has a physiological effect up to a distance of 100 μm . Overall this study provides new information on cellular mechanisms regulating growth cone motility, and suggests a potential role of PI-3K and NO in neuronal pathfinding *in vivo*.

INDEX WORDS: Growth cone, Filopodia, Phosphatidylinositol-3-kinase, Nitric oxide, Filamentous actin, Akt, RhoA activated kinase, MEK, *Helisoma trivolvis*

SIGNALING MECHANISMS REGULATING NEURONAL
GROWTH CONE DYNAMICS

by

KARINE TORNIERI

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy
in the College of Arts and Sciences
Georgia State University

2008

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Karine Tornieri
2008

SIGNALING MECHANISMS REGULATING NEURONAL
GROWTH CONE DYNAMICS

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Georgia State University
December 2008

DEDICATION

To my grand father,

A Papi,

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I would like to express gratitude to my advisor Dr Vincent Rehder for his great guidance during these years. I have learned so much working with you; you taught me how to design an experiment, especially doing the right controls. You let me being independent, but at the same time you were always there to answer my questions and guide me. You are a wonderfull advisor, but also a really great person to know and be around, thank you again for helping me to become the scientist that I am.

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LIST OF ABBREVIATIONS

| | |
|----------------------------------|--|
| AA | Arachidonic acid |
| ABPs | actin binding proteins |
| ADF | actin depolymerizing factor |
| BDNF | brain derived neurotrophic factor |
| Bleb | blebbistatin |
| Ca ²⁺ | calcium |
| [Ca ²⁺] _i | intracellular calcium concentration |
| Ca ²⁺ /CaM | calcium/calmodulin |
| cADPR | cyclic adenosine diphosphate ribose |
| CAM | cell adhesion molecule |
| CaM | calmodulin |
| CaMKII | calcium calmodulin dependent protein kinase II |
| cAMP | cyclic adenosine monophosphate |
| cGMP | cyclic guanosine monophosphate |
| CM | conditioned medium |
| CNG | cyclic nucleotide gated |
| CNS | central nervous system |
| Cyto B | cytochalasin B |
| DM | defined medium |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DRG | dorsal root ganglion |

| | |
|---------|-------------------------------------|
| ECM | extracellular matrix |
| EGF | epidermal growth factor |
| eNOS | endothelial nitric oxide synthase |
| ET | esophageal nerve trunk |
| F-actin | filament actin |
| GAF | GTPase activating factors |
| GAP-43 | growth associated protein 43 |
| GEF | guanine nucleotide exchange factors |
| GDP | guanosine diphosphate |
| GPI | glycosylphosphatidylinositol |
| GTP | guanosine triphosphate |
| Ig | immunoglobulin |
| iNOS | inducible nitric oxide synthase |
| IP3 | inositol-3 phosphate |
| KCl | potassium chloride |
| L-15 | leibovitz's L-15 medium |
| LIMK | LIM kinase |
| MAG | myelin associated glycoprotein |
| MAPK | mitogen activated protein kinase |
| MEK | MAP kinase kinase |
| MLCK | myosin light chain kinase |
| NCAM | neuronal cell adhesion molecule |
| NT | neurotrophin |

| | |
|-------------------|-----------------------------------|
| NGF | nerve growth factor |
| nNOS | neuronal nitric oxide synthase |
| NOS | nitric oxide synthase |
| NO | nitric oxide |
| N-WASP | wiskott-aldrich syndrome protein |
| ONOO ⁻ | peroxynitrite |
| PAK | p21-activated kinase |
| PDEIII | phosphodiesterase III |
| PI | phosphatidylinositol |
| PIP ₂ | phosphatidylinositol-biphosphate |
| PIP ₃ | phosphatidylinositol-triphosphate |
| PI-3K | phosphatidylinositol-3-kinase |
| PI-4K | phosphatidylinositol-4-kinase |
| PKC | protein kinase C |
| PKG | protein kinase G |
| PLA ₂ | phospholipase A ₂ |
| RNS | reactive nitrogen species |
| ROCK | RhoA activated protein kinase |
| RPeD1 | right pedal dorsal 1 |
| RyR | ryanodine receptor |
| sGC | soluble guanylyl cyclase |
| Sema3A | semaphorin 3A |
| Trk | tropomyosin related kinase |

| | |
|------|------------------------------|
| TRP | transient receptor potential |
| Wort | wortmannin |

Chapter 1 : General Introduction

Specific Aims of Dissertation

Growth cones located at the tip of newly extended neurites are critical motile and sensory structures that are necessary for correct neuronal guidance during the development and regeneration of the nervous system. Filopodia, composed of bundles of F-actin, are the first sites of contact with guidance cues, and contact of a single filopodium is enough to reorient the growth cone toward or away from the contact point. The actin-based motility of growth cone filopodia is integral to correct guidance and subsequent connectivity. Therefore a better understanding of molecules and second messengers that regulate filopodial dynamics is crucial to prevent neuronal misguidance during the development or regeneration of the nervous system. The objective of this study is to determine the cellular mechanisms and second messengers involved in the regulation of growth cone dynamics. This dissertation employs *Helisoma trivolvis* as a model system to investigate: (i) the role of PI-3K and its downstream targets in regulating growth cone dynamics, and (ii) the effect of physiological concentrations of the gaseous messenger NO on growth cone morphology.

Specific Aim 1 (Chapter 2): Does PI-3K and its downstream targets regulate filopodial dynamics?

PI-3K has been demonstrated to regulate neurite outgrowth and to be expressed in growth cones (Rodgers and Theibert, 2002). These data suggest that PI-3K can regulate growth cone guidance, but does not provide information about the pathway through which PI-3K exerts its effect, or its role in the regulation of growth cone morphology. Therefore the present study tests the hypothesis that PI-3K affects filopodial dynamics and neurite outgrowth. Furthermore, this study hypothesizes that these effects are mediated through the lipid and protein kinase activities of PI-

3K, and subsequently via Akt, Rho kinase (ROCK) and MEKK, ultimately affecting the dynamics of the actin cytoskeleton.

Specific Aim 2 (Chapter 3): Determine whether NO release from a physiological source affects growth cone motility and examine the underlying signaling pathway.

Global and local applications of NO via NO donors on *Helisoma* B5 neurons induce an increase in filopodial length, a decrease in filopodial number and an increase in $[Ca^{2+}]_i$ (Van Wagenen and Rehder, 1999; Van Wagenen and Rehder, 2001; Welshhans and Rehder, 2005; Welshhans and Rehder, 2007). Local application of NO exerts its effect on growth cone morphology and calcium concentration via a signaling pathway involving sGC, cyclic guanosine monophosphate (cGMP), protein kinase G (PKG), and cyclic adenosine diphosphate ribose (cADPR) (Welshhans and Rehder, 2005; Welshhans and Rehder, 2007). These data suggest that NO can regulate growth cone dynamics during neuronal pathfinding. However the effect of NO release from a physiological source on growth cone morphology has never been investigated. The present study tests the hypothesis that NO which is spontaneously released from a single cell can affect growth cone dynamics of neighboring neurons in a paracrine fashion. Such a demonstration would provide evidence for NO acting as a paracrine messenger with the potential to affect the outcome of neuronal pathfinding.

Overall, this dissertation investigates cellular mechanisms that control growth cone morphology during neuronal pathfinding. Identification of molecules and second messengers that affect growth cone dynamics has implications for clinical strategies to enhance correct

navigation and appropriate connectivity during development of the nervous system, and also during regeneration after injury.

The complex wiring and rewiring of the nervous system during development and regeneration following injury require newly extended neurites to navigate long distances in order to reach their targets and form synapses. This neuronal pathfinding depends on a sensory and dynamic structure located at the tip of extending neurites, called the growth cone, on which spatially and temporally regulated extracellular cues bind to receptors and trigger intracellular signaling cascades that regulate cytoskeletal dynamics (Huber et al., 2003; Gallo and Letourneau, 2004). Many of these guidance cues and their receptors have now been identified; the guidance cues have been classified into two main categories by their action on growth cones, attractive or repulsive (Goodman, 1996; Tessier-Lavigne and Goodman, 1996; Huber et al., 2003). In each category these cues can have an effect over a short distance (contact-dependent attraction or repulsion) or over a long distance (chemoattraction or chemorepulsion) (Goodman, 1996; Tessier-Lavigne and Goodman, 1996; Huber et al., 2003). However, it has recently been shown that many individual cues can be both attractive and repulsive, depending (i) on the state of the growth cone, (ii) on the expression of specific receptors and (iii) cell type (Huber et al., 2003; Nishiyama et al., 2003; Bartoe et al., 2006; McKenna et al., 2008). Although studies on extracellular cues and their effects on neuronal guidance have been well documented, less is known about the intracellular signaling mechanisms that regulate growth cone behavior. Thus, investigating signaling pathways and the integration of different signals in the growth cone will allow for a better understanding of mechanisms controlling neuronal pathfinding during development and regeneration of the nervous system.

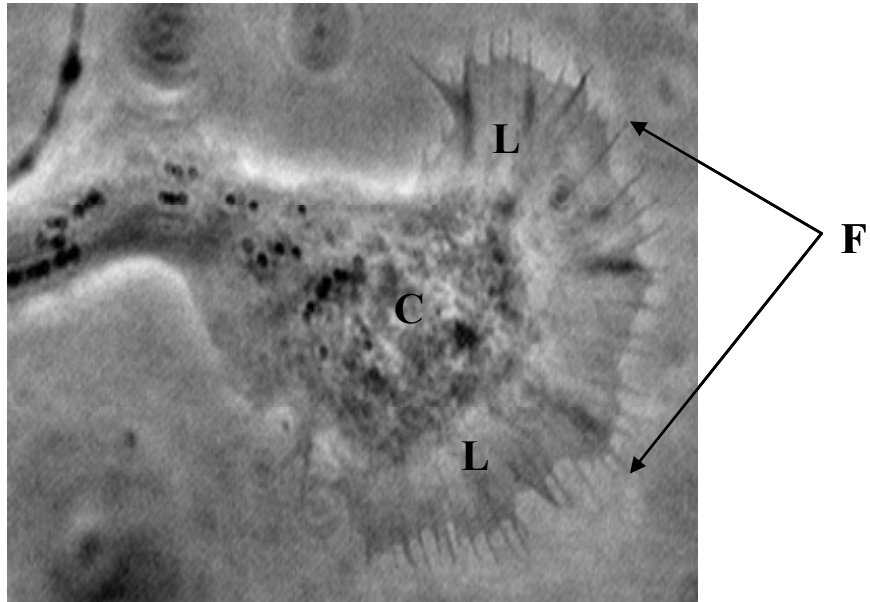
Growth Cone Structure and Dynamics

Growth cones, located at the tip of extending neurites, were observed in fixed chick embryonic tissues for the first time in the late 1800s by the Spanish neuroanatomist Ramon y Cajal. He described growth cones as “battering rams” finding their way through many obstacles to reach their targets, and already envisioned a guidance mechanism involving attractive gradients of diffusible cues released from their targets (Cajal, 1890). In 1910, growth cones were observed in embryonic neuronal tube explants by Ross Harrison (Harrison, 1910), and *in vivo* on growing nerve fibers in the transparent tail of a frog by Speidel in 1930 (Speidel, 1941). It has been shown that growth cones, isolated from their cell bodies *in vivo*, are still able to grow and navigate correctly to their final destinations (Bray et al., 1978; Harris et al., 1987), suggesting that growth cones themselves contain the machinery to sense and respond to guidance cues.

The growth cone is composed of three regions: (i) the central domain, which contains microtubules and organelles, (ii) the lamellipodium, composed of a meshwork of filamentous actin (F-actin), and (iii) finger like extensions, called filopodia, made of bundles of F-actin (Smith, 1988; Huber et al., 2003) (Fig. 1.1). Guidance cues bind to receptors located on the membrane of filopodia, which continuously extend and retract to explore their environment. This area which is surveyed by the growth cone at any given point in time is called its ‘action radius’. Therefore, the ability of a growth cone to be dynamic is critical for correct pathfinding and application of drugs that interfere with filopodial dynamics result in pathfinding errors (Bentley and Toroian-Raymond, 1986; Chien et al., 1993). During the journey toward their targets, growth cone morphology and the speed of neurite advance changes at various points along their path (Tosney and Landmesser, 1985; Bovolenta and Mason, 1987).

Figure 1.1: Phase contrast image of a *Helisoma trivolvis* B5 growth cone.

The growth cone contains 3 domains, the central domain (C), the lamellipodia (L), and filopodia (representative filopodia indicated by the arrows).



Growth cones fasciculating with pioneer axons have a simple bullet shape and grow faster, whereas growth cones reaching decision points become more complex, have more filopodia and lamellipodia and advance slower. Once growth cones reach their target region, their shape becomes thinner with many branches (Harris et al., 1987). As mentioned above, filopodia are the sensing devices of the growth cone and it has been shown that removal of filopodia will not prevent a growth cone from advancing but will affect correct navigation (Bentley and Toroian-Raymond, 1986; Chien et al., 1993). The importance of filopodia, and more specifically of single filopodia, in growth cone guidance has been demonstrated *in vitro* and *in vivo*. For example, contact of a single filopodium with a laminin-coated area in the culture dish or a guidepost cell results in the reorientation of the growth cone toward the contact point (O'Connor et al., 1990; Letourneau, 1996). In *Helisoma* neurons, stimuli known to induce an increase in the intracellular calcium concentration and/or changes in filopodial length, will cause the same effect on isolated filopodia, suggesting that filopodia can act as autonomous units (Davenport et al., 1993).

What are the mechanisms controlling growth cone dynamics? Pharmacological studies identify F-actin as a key player in growth cone motility (Marsh and Letourneau, 1984; Letourneau et al., 1987) F-actin is polymerized in growth cones from G-actin monomers that are added at the plus end (barbed end) of F-actin at the leading edge of filopodia and lamellipodia. Actin filaments are very dynamic, involving actin polymerization at the plus end, retrograde flow toward the central domain (Zhou and Cohan, 2004), actin depolymerization at the minus end, and recycling of actin subunits for further actin polymerization (Lin et al., 1996; Mallavarapu and Mitchison, 1999; Jay, 2000; Suter and Forscher, 2000). Actin turnover has been shown to be critical for growth cone motility during pathfinding. Removal of filopodia on T11 pioneer growth

cones in the grasshopper limb *in vivo* by treatment with the actin depolymerizing agent, cytochalasin, induced misguidance (Bentley and Toroian-Raymond, 1986). Errors in pathfinding after treatment with cytochalasin were also observed in the *Xenopus* visual system, with retinal axons missing the tectum (Chien et al., 1993). These experiments, in addition to emphasizing a role of actin turnover in growth cone motility, suggest that filopodia are not necessary for neurite advance itself, but for correct pathfinding in response to guidance cues. The rate of filopodial elongation depends on both the rate of F-actin polymerization and retrograde flow (Mallavarapu and Mitchison, 1999). During pathfinding, growth cones will exhibit a variety of behaviors, such as collapse, and turning toward or away from gradients of guidance cues. These behaviors involve reorganization of the actin cytoskeleton in addition to regulation of actin turnover. Growth cones exposed to an attractive cue will have more and longer filopodia in the direction of the cue (Bentley and O'Connor, 1994; Zheng et al., 1996; Gallo et al., 1997), creating an asymmetrical cytoskeletal organization, which is critical for growth cone steering. Indeed, depolymerization of F-actin on one side of a growth cone with a local gradient of cytochalasin induces turning of the growth cone away from the gradient (Yuan et al., 2003).

Microtubules have been recently suggested to be more than a secondary player in growth cone pathfinding (structural support for neurite advance), but instead to have a critical role in this process (Dent and Kalil, 2001; Gordon-Weeks, 2004; Kalil and Dent, 2005). In the axon, microtubules are organized in bundles and splay out once they enter the base of the growth cone, where a few invade the lamellipodium and some may even enter individual filopodia (Tsui et al., 1984; Gordon-Weeks, 1991; Sabry et al., 1991; Zhou and Cohan, 2004). Microtubules, like F-actin, are dynamic polymers, switching between phases of growth and shrinkage, with tubulin polymerization occurring at the plus end, which points toward the F-actin meshwork in the

peripheral domain (Heidemann et al., 1981; Kirschner and Mitchison, 1986; Baas et al., 1988). Pharmacological approaches have suggested a role for microtubule dynamics in growth cone steering. Pretreatment of *Xenopus* spinal neurons with taxol or nocodazole, drugs known to stabilize or destabilize microtubules, respectively, blocks the turning effect of glutamate and netrin-1. Local stabilization of microtubules on one side of *Xenopus* growth cones induces steering toward that side, whereas local destabilization induces turning away from the side exposed to nocodazole (Buck and Zheng, 2002). The similarity between the effects of blocking actin and microtubule dynamics, and the observation of interactions between these two polymers in the lamellipodium and individual filopodia suggest a possible interaction between microtubules and F-actin during growth cone steering by guidance cues.

Guidance Cues and the Molecular Machinery of Growth Cones

Rho GTPases and actin dynamics

Studies on fibroblasts have identified small GTP-binding proteins of the Rho subfamily (Rho GTPases), such as Cdc42, Rac and Rho, as critical players in the formation of filopodia, lamellipodia, focal adhesions and stress fibers, respectively (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995; Mueller, 1999). Injection of active Cdc42 into fibroblasts induced filopodial formation, whereas active Rac induced lamellipodial formation (Ridley et al., 1992; Kozma et al., 1995; Nobes and Hall, 1995). Development of stress fibers and focal adhesions were observed after injection of active Rho (Ridley and Hall, 1992; Nobes and Hall, 1995). Furthermore, in the nervous system, Rho GTPases are temporally expressed during axonal outgrowth in *Drosophila*, rats and chicken (Luo et al., 1994; Malosio et al., 1997; Threadgill et al., 1997). Additionally, RhoA effectors promote growth cone collapse and neurite retraction

(Wahl et al., 2000; Gallo and Letourneau, 2004). Rac-1 and Cdc42 have been found to regulate filopodial and lamellipodial dynamics in growth cones, and neurite guidance (Kuhn et al., 1998; Brown et al., 2000). During neuronal pathfinding, guidance cues bind and activate receptors on filopodia, which in turn regulate growth cone actin dynamics by activating Rho GTPases (Gallo and Letourneau, 1998; Dickson, 2001; Huber et al., 2003; Gallo and Letourneau, 2004). The activity state of Rho GTPases is determined by the binding of guanine nucleotides. Binding of GTP to Rho GTPases activates them, whereas GDP inactivates them. Two main classes of proteins control Rho GTPases activity, guanine nucleotide exchange factors (GEF) and GTPase activating factors (GAF). GEFs facilitate the exchange of GDP to GTP, which in turn activate Rho GTPases, whereas GAFs turn off their signaling by enhancing the endogenous GTPase activities of Rho GTPases (Hall, 1994; Dickson, 2001; Huber et al., 2003).

Rho GTPases integrate signaling from many guidance cues, leading to specific growth cone behaviors such as turning, branching, and collapse by controlling actin polymerization, branching, actin depolymerization and retrograde flow. Recent studies have unraveled the mechanisms by which Rho GTPases regulate actin dynamics. Cdc42 and Rac induce actin nucleation and branching by activating the Arp2/3 complex through the effectors neuronal Wiskott-Aldrich Syndrome protein (N-WASP) and SCAR/WAVE, respectively (Machesky et al., 1999; Rohatgi et al., 1999). Actin polymerization is controlled by (i) the protein profilin, which binds to G-actin monomers, facilitating their addition at the plus end of F-actin and (ii) its effectors, proteins from the VASP/Ena family, which in addition to activating profilin also associate with the plus end of F-actin and prevent the binding of capping proteins (Suetsugu et al., 1998; Bear et al., 2002). Actin depolymerization at the minus end involves the F-actin severing protein, cofilin. The activity of cofilin is regulated by phosphorylation and

dephosphorylation, with inactivation induced by phosphorylation through LIM kinase (LIMK) (Arber et al., 1998; Yang et al., 1998), and activation caused by dephosphorylation via the phosphatase Slingshot (Niwa et al., 2002). LIMK is activated by p21-activated kinase (PAK), the effector of Cdc42 and Rac, and by ROCK, the effector of RhoA (Arber et al., 1998; Yang et al., 1998). Rho GTPases control F-actin retrograde flow by regulating non-muscle myosin via phosphorylation by ROCK or myosin light chain kinase (MLCK), the latter being inactivated by PAK, the effector of Rac and Cdc42 (Amano et al., 1996; Sanders et al., 1999). To summarize, during neuronal pathfinding, guidance cues can regulate growth cone behavior through RhoGTPase signaling, which mediates the activation of actin binding proteins (ABPs).

Guidance cues and RhoGTPases

During development of the nervous system, guidance cues guide extending neurites by regulating growth cone actin dynamics. However, it remains to be determined which RhoGTPases mediate the effect of particular guidance cues. In this section I will briefly introduce only the guidance cues known to mediate their effect via RhoGTPase signaling.

The diffusible factor netrin has been shown to act bifunctionally in vertebrates, being attractive for spinal cord commissural axons to the floor plate and repulsive for trochlear motor axons (Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995). Furthermore, netrin receptors have been identified: attractive receptors are UNC-40, DCC and Frazzled in *C.elegans*, vertebrate and *Drosophila* respectively (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996), whereas UNC-5 and UNC 40 have been shown to mediate repulsion in *C. elegans* (Amano et al., 1996; Chan et al., 1996; Ackerman et al., 1997; Leonardo et al., 1997; Mueller, 1999). In vertebrates, netrin-1-DCC signaling involves the RhoGTPases, Rac and Cdc42.

Coexpression of DCC, and dominant negative forms of Rac and Cdc42 in HEK 293T cells or neuroblastoma cells blocks the effect of netrin-1 on filopodial number and cell morphology (Shekarabi and Kennedy, 2002). Furthermore, Rac1 and Cdc42 activities have been shown to be necessary for DCC-induced neurite outgrowth in N1E-115 neuroblastoma cells (Li et al., 2002). Netrin-1 affects growth cone dynamics via cyclic nucleotides, and the intrinsic level of cyclic nucleotides modulates the effect of netrin-1. For example, experiments using growth cone turning assays indicated that high levels of cyclic adenosine monophosphate (cAMP) mediated netrin-1 induced attraction, whereas low levels of cAMP mediated netrin-1 induced repulsion of embryonic *Xenopus* spinal neurons (Ming et al., 1997; Song et al., 1997).

Semaphorins, which can be both diffusible and membrane bound, can also act as either attractive or repulsive guidance cues (Messersmith et al., 1995; Shepherd et al., 1997; Varela-Echavarria et al., 1997). *In vitro* studies on chick dorsal root ganglion neurons suggest a role for Rac1 in mediating collapsin1- or semaphorin3A-induced growth cone collapse (Jin and Strittmatter, 1997). Activation of RhoA seems to be critical for semaphorin4-induced growth cone collapse (Huber et al., 2003). Similar to netrin, semaphorin signaling involves cyclic nucleotides. Indeed, cyclic guanosine monophosphate (cGMP) modulates the effect of semaphorin3 on growth cone motility, because growth cone repulsion can be converted to attraction by increasing the intracellular level of cGMP (Song and Poo, 1999).

Another type of repulsive cue, ephrins, which bind to their tyrosine kinase receptors Ephs, play a role in the formation of topographic maps (Cheng et al., 1995; Drescher et al., 1997), patterning of forebrain and hindbrain (Xu et al., 1995; Xu et al., 1996), and the formation of certain commissures (Orioli et al., 1996; Park et al., 1997). EphA receptors interact with glycosylphosphatidylinositol (GPI) anchored ephrin A ligands, whereas EphB receptors bind to

ephrin B transmembrane ligands (Frisen and Barbacid, 1997). A particularity of these ligands is that their signaling is bidirectional, meaning that binding of ligands to receptors triggers signaling in the cell expressing the receptor (forward signaling), as well as in the ligand-expressing cell (backward signaling) (Kullander and Klein, 2002). Ephrin signaling involves RhoGTPase activation; for example, ephrin-A5 has been shown to induce growth cone collapse of retinal ganglion cells through activation of RhoA and its effector ROCK (Wahl et al., 2000). Furthermore, retinal axon retraction in response to ephrin-A2 involves RhoA and Rac1 signaling (Gallo et al., 2002; Journey et al., 2002).

Neurotrophins, such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and neurotrophin 3 and 4 (NT-3 and NT-4) have been well documented to play a role in neuronal growth and survival during the development of the nervous system *in vivo* (Huang and Reichardt, 2001); however, recent studies *in vitro* suggest that they also have the ability to act as potent guidance cues. For example, NGF induces attractive turning of chick dorsal root and retinal axons (Gundersen and Barrett, 1979; Gallo et al., 1997). In addition, these four neurotrophins have been shown to induce growth cone turning of *Xenopus* spinal neurons *in vitro* (Song et al., 1997; Ming et al., 1999). Neurotrophins bind with high affinity to tropomyosin related kinase (Trk) receptors, which are tyrosine kinase receptors. NGF binds to TrkA, BDNF to TrkB and NT-3 to TrkC. Stimulation of TrkA by NGF induces the activation of Cdc42 and Rac, as well as the inactivation of RhoA (Sebok et al., 1999; Yamaguchi et al., 2001; Nusser et al., 2002). Inhibition of the RhoA effector, ROCK, blocks NGF induced growth cone steering (Loudon et al., 2006). These results strongly suggest a role for RhoGTPases in NGF-mediated regulation of neurite outgrowth. Neurotrophins also bind with low affinity to another type of receptor, the p75 receptor. P75 has been suggested to bind RhoA directly and to inactivate it

upon binding of neurotrophins (Yamashita et al., 1999). Indeed, BDNF induces filopodial elongation on chick retinal growth cones by inactivating RhoA through p75 stimulation (Gehler et al., 2004). As observed with netrin-1 and semaphorin, the attractive effect of BDNF and NGF can be converted to repulsion by decreasing the cAMP level (Song et al., 1997).

As mentioned earlier, neurites can be guided by short range guidance cues through contact with cues in the extracellular matrix (ECM), or cell adhesion molecules (CAM) expressed on both pathfinding neurons and neighboring cells (Tessier-Lavigne and Goodman, 1996). Cell adhesion receptors include immunoglobulin (Ig) family members such as NCAM, as well as cadherins and integrins. Binding of these receptors to ligands in the ECM or to CAM on neighboring cells triggers signaling pathways that affect neurite outgrowth. Mitogen activated protein kinase (MAPK) pathways have been well documented to mediate cell adhesion receptor signaling (Tessier-Lavigne and Goodman, 1996; Huber et al., 2003). In addition to MAPK pathways, integrins and cadherins can also activate RhoGTPase signaling (Clark et al., 1998; Kaibuchi et al., 1999; Ren et al., 1999).

Role of the Second Messenger Calcium in Axonal Guidance

Importance of calcium in growth cone motility and steering

Calcium (Ca^{2+}) is an important second messenger that relays guidance cues to regulate growth cone motility and steering (Song and Poo, 1999; Gomez and Spitzer, 2000; Henley and Poo, 2004). The intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) in the growth cone is regulated by several mechanisms, such as influx through plasma membrane Ca^{2+} channels, release from internal stores (mitochondria and endoplasmic reticulum) through inositol-3 phosphate (IP3) and ryanodine sensitive channels, and extrusion through plasma membrane adenosine triphosphate

dependent pumps and sodium-calcium exchangers (Lipscombe et al., 1988; Clapham, 1995; Henley and Poo, 2004; Wen and Zheng, 2006). Calcium has been shown to play a role in neurite outgrowth, but early studies from different research groups were not consistent and sometimes even conflicting. Some groups demonstrated that Ca^{2+} facilitated neurite outgrowth (Nishi and Berg, 1981; Suarez-Isla et al., 1984) while others suggested that Ca^{2+} prevented it (Bixby and Spitzer, 1984; Mattson and Kater, 1987; Silver et al., 1989). An explanation for this range of responses was provided by the hypothesis of a “ Ca^{2+} set point” (Kater and Mills, 1991) stating that within an optimal range of $[\text{Ca}^{2+}]_i$ neurites grow at their maximal speed, but that changes of $[\text{Ca}^{2+}]_i$ below or above the optimal range will slow down or stop growth cone motility. In addition to regulating neurite outgrowth, $[\text{Ca}^{2+}]_i$ plays a critical role in growth cone steering. Application of an electric field affects the speed of neurite advance of *Xenopus* spinal neurons and mouse dorsal root ganglion neurons and induces growth cone steering toward the cathode (Hinkle et al., 1981; Patel and Poo, 1982; Fields et al., 1990). Interestingly, the effect of electric fields on growth cone motility is mediated by a local increase in $[\text{Ca}^{2+}]_i$ (Bedlack et al., 1992; Davenport and Kater, 1992).

Guidance cue-induced growth cone steering, such as seen with netrin-1, NGF, BDNF and myelin associated glycoprotein (MAG), also requires Ca^{2+} signaling (Song et al., 1997; Hong et al., 2000; Henley et al., 2004). For example, a gradient of netrin-1 has been shown to induce growth cone attraction by increasing $[\text{Ca}^{2+}]_i$ via calcium influx through voltage dependent Ca^{2+} channels (Hong et al., 2000; Niwa et al., 2002; Nishiyama et al., 2003). Furthermore, recent studies have demonstrated a role of transient receptor potential (TRP) channels in netrin-1, BDNF and MAG induced growth cone steering (Li et al., 2005; Shim et al., 2005; Wang and Poo, 2005). Growth cone steering requires an asymmetric increase of $[\text{Ca}^{2+}]_i$ in the growth cone.

For example, local increases of $[Ca^{2+}]_i$ in *Xenopus* and dorsal root ganglion growth cones by using a gradient of ryanodine or Ca^{2+} ionophore respectively, is sufficient to induce growth cone steering (Gundersen and Barrett, 1980; Hong et al., 2000). These results are further supported by experiments using focal photolysis of caged Ca^{2+} to induce growth cone turning (Zheng, 2000). Surprisingly a local increase in $[Ca^{2+}]_i$ in growth cones induces both attraction and repulsion, depending on the amplitude of the increase. Focal photolysis of caged Ca^{2+} in the presence of extracellular Ca^{2+} induces attraction of growth cones, whereas when the resting level of $[Ca^{2+}]_i$ is decreased in the absence of extracellular Ca^{2+} , the same focal release induces repulsion (Zheng, 2000). Gradients of netrin-1 and MAG induce growth cone attraction and repulsion, respectively, by increasing $[Ca^{2+}]_i$ on the side of the gradient. Netrin-1 causes a higher increase in $[Ca^{2+}]_i$ than MAG, mediated by both Ca^{2+} influx through plasma membrane Ca^{2+} channels and Ca^{2+} -induced Ca^{2+} release from internal stores, whereas MAG acts through Ca^{2+} release from internal stores only (Hong et al., 2000; Nishiyama et al., 2003; Henley et al., 2004). To summarize, a global increase in $[Ca^{2+}]_i$ affects neurite outgrowth, whereas a local increase mediates growth cone steering.

It is also important to note that transient increases in $[Ca^{2+}]_i$ localized to filopodia have been observed during neurite outgrowth *in vitro*, with the local increase in $[Ca^{2+}]_i$ propagating to the central domain, and leading to a global increase in $[Ca^{2+}]_i$ (Gomez et al., 2001). Experiments in our laboratory have investigated the effect of spatio-temporal increases in $[Ca^{2+}]_i$ on filopodial dynamics of *Helisoma* B5 neurons. Global and regional uncaging of Ca^{2+} in the growth cone induced a global increase in filopodial length, whereas local release within an individual filopodium induced elongation that was restricted to the stimulated filopodium (Cheng et al.,

2002). These results suggest a critical role for spatio-temporal Ca^{2+} signaling in neuronal pathfinding.

Calcium signaling

The effect of calcium signaling on neurite outgrowth and growth cone steering is mediated by changes in cytoskeleton dynamics. A well documented effector of Ca^{2+} is the Ca^{2+} binding protein, calmodulin (CaM). CaM is highly expressed in filopodia and the central domain of growth cones, and is sensitive to $[\text{Ca}^{2+}]_i$ just above basal calcium levels (Zucker, 1999). Inhibition of CaM induces mistakes in pathfinding *in vivo* (VanBerkum and Goodman, 1995), and prevents laminin-induced growth cone attraction *in vitro* (Kuhn et al., 1998). Calcium-calmodulin activates calcium/calmodulin-dependent kinases (CaMK) and the phosphatase calcineurin (Henley and Poo, 2004). Uncaged Ca^{2+} -induced filopodial elongation in *Helisoma* B5 neurons is mediated by activation of CaM and calcineurin (Cheng et al., 2002). Furthermore, recent studies in *Xenopus* and other organisms have shown that overexpression of active CaMKII facilitates neurite outgrowth, whereas inhibition prevents it (Goshima et al., 1993; Kuhn et al., 1998; Zou and Cline, 1999). Calcineurin also prevents neurite outgrowth in *Xenopus* spinal neurons and promotes it in chick dorsal root ganglion neurons (Chang et al., 1995; Lautermilch and Spitzer, 2000). Another protein mediating Ca^{2+} signaling is protein kinase C, which is activated by calcium and phosphorylates a number of proteins, including GAP-43 (Liu and Storm, 1990). Phosphorylation of GAP-43 by PKC results in the dissociation of GAP-43 from CaM, allowing free CaM to be activated by Ca^{2+} (Liu and Storm, 1990). It is important to note that phosphorylated GAP-43 stabilizes F-actin, whereas dephosphorylation by calcineurin destabilizes it (He et al., 1997). Furthermore, PKC has been shown to mediate growth cone

turning in *Xenopus* spinal neurons and growth cone dynamics in *Helisoma* B5 neurons (Cheng et al., 2000; Xiang et al., 2002). Another downstream protein of Ca^{2+} signaling is myosin II, which is activated by phosphorylation via Ca^{2+} /CaM dependent myosin light chain kinase and/or by ROCK (Amano et al., 1996; Dent and Gertler, 2003), and inactivated by RhoGTPases, Cdc42 and Rac (Sanders et al., 1999). To summarize, calcium is a critical second messenger regulating growth cone dynamics during neuronal pathfinding.

Phosphatidylinositol-3-Kinase (PI-3K)

PI-3K signaling

PI-3K has been implicated in a variety of cellular activities, including cell survival, mitogenesis, and differentiation (Krasilnikov, 2000; Rodgers and Theibert, 2002). There are three classes of PI-3K: Class I contains a p110 catalytic subunit and a regulatory adapter subunit, and preferentially phosphorylates phosphatidylinositol 4,5 biphosphate [PtdIns(4,5)P₂] to PtdIns(3,4,5)P₃ (Vanhaesebroeck and Waterfield, 1999; Cantrell, 2001). Class II phosphorylates PtdIns and PtdIns(4)P to PtdIns(3)P and PtdIns(3,4)P₂ respectively, whereas class III phosphorylates only PtdIns to PtdIns(3)P (Vanhaesebroeck and Waterfield, 1999; Cantrell, 2001). Class I PI-3K can be activated by receptor- and non-receptor tyrosine kinases, as well as by G-protein coupled receptors (Stephens et al., 1994; Stoyanov et al., 1995; Lopez-Illasaca et al., 1997; Duronio et al., 1998; Vanhaesebroeck and Waterfield, 1999; Cantrell, 2001). PI-3K, in turn, has two modes of action. First of all, it functions as a lipid kinase by phosphorylating the 3'-OH position on the inositol ring of several phosphoinositides, thereby producing inositol lipid products, which are important mediators of intracellular signaling (Duronio et al., 1998; Vanhaesebroeck and Waterfield, 1999; Cantrell, 2001). This signaling pathway leads to the

stimulation of guanine nucleotide exchange factors for Rho-GTPases, as well as to the activation of the PH-domain containing kinase, PDK-1, which in turn activates Akt (Alessi et al., 1997; Downward, 1998; Meier and Hemmings, 1999; Vanhaesebroeck and Alessi, 2000). Secondly, PI-3K functions as a protein kinase resulting in the activation of the Ras/Raf/Erk and Cdc42/Rac pathways (Bondeva et al., 1998; Vanhaesebroeck and Waterfield, 1999; Krasilnikov, 2000). Both the lipid and the protein kinase activities of PI-3K regulate downstream targets known to affect actin cytoskeletal organization (Rodgers and Theibert, 2002), suggesting a potential role of PI-3K in growth cone dynamics.

Role of PI-3K in the nervous system

Developing and adult rat brains express PI-3K (Ito et al., 1995; Bartlett et al., 1999; Rodgers and Theibert, 2002) and upregulation of PI-3K has been observed after axonal crush and ischemia (Ito et al., 1996; Kitagawa et al., 1999). An increase in PI-3K activity and protein level occurs in amyotrophic lateral sclerosis (Wagey et al., 1998), whereas a decrease in PI-3K activity is observed in Alzheimer's disease (Zubenko et al., 1999). The function of PI-3K in neuronal survival is well established (Yao and Cooper, 1995; Rodgers and Theibert, 2002). In addition to its involvement in mediating cell survival, PI-3K has been reported to stimulate neurite outgrowth in PC12 cells (Kita et al., 1998), to mediate ephrin A5 and slit-2 induced growth cone collapse in chick retinal ganglion cells (Wong et al., 2004), and to be required for the NCAM-mediated neurite outgrowth of primary neurons (Ditlevsen et al., 2003). In addition, PI-3K has been shown to play a role in lamellipodial formation of PC12 cells (Posern et al., 2000) and in the regulation of neuronal polarity (Shi et al., 2003; Menager et al., 2004). These effects are likely mediated by changes at the level of the neuronal cytoskeleton. The mechanism by which

PI-3K affects neurite outgrowth is presently unclear, but prominent targets downstream of PI-3K include Akt (Protein kinase B), regulators of Rho-GTPase family members, and the RAS/RAF/ERK pathway (Corvera and Czech, 1998; Duronio et al., 1998; Vanhaesebroeck and Waterfield, 1999; Bishop and Hall, 2000; Krasilnikov, 2000; Cantrell, 2001). Studies have shown that expression of a dominant inhibitory form of Akt increases NGF-induced neurite outgrowth in PC12 cells (Bang et al., 2001; Piiper et al., 2002). Yet, pharmacological inhibition of PI-3K reduces neurite outgrowth in mouse DRG and hippocampal neurons (Wu et al., 1998; Edstrom and Ekstrom, 2003), as well as in PC12 cells (Jackson et al., 1996). Furthermore, PI-3K activation by NGF has been shown to increase axon outgrowth in DRG neurons by inactivation of GSK-3 β and subsequent involvement of the microtubule plus end binding protein APC (Zhou et al., 2004), although NGF stimulation has also been reported to signal via the MAPK pathway (Goold and Gordon-Weeks, 2005). To summarize PI-3K may regulate neurite outgrowth via Akt and MAPK pathway.

Role of the Gaseous Second Messenger Nitric Oxide (NO) in the Nervous System

NO signaling

NO is a gas produced by the enzyme nitric oxide synthase (NOS), which is known to have 3 isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) (Garthwaite, 1991). Activation of cells containing iNOS, such as macrophages, microglia and astrocytes, during disease and injury leads to the production of NO (Hall, 1994; Cassina et al., 2002). Like other free radicals, NO in high concentrations can be toxic due its formation of reactive nitrogen species (RNS) such as peroxynitrite (ONOO⁻), via the reaction of nitric oxide and superoxide (Lewen et al., 2000). A main target of NO is the enzyme soluble guanylyl cyclase (sGC), which

once activated by the binding of NO to its heme moiety (Dierks and Burstyn, 1996), forms cGMP. cGMP can increase $[Ca^{2+}]_i$ by activating PKG, which in turn can activate ryanodine-sensitive calcium stores located in the endoplasmic reticulum (Willmott et al., 1996; Looms et al., 2001; Vicente et al., 2005). cGMP can also increase $[Ca^{2+}]_i$ by activating cyclic nucleotide-gated ion (CNG) channels located in the plasma membrane. This is achieved by cGMP elevating the concentration of cAMP through inhibition of phosphodiesterase III (PDEIII) (Houslay and Milligan, 1997; Tsukada et al., 2002; Jensen et al., 2004). Recent studies have reported a role for Ca^{2+} /Calmodulin in the formation of NO through activation of some NOS isoforms (Bredt and Snyder, 1990; Cho et al., 1992; Li and Poulos, 2005). Other mechanisms of NO action are exerted via nitrosylation of cysteine residues in proteins to produce S-Nitrosothiols (Arnold et al., 1977; Stamler et al., 1992; Jaffrey et al., 2001; Ahern et al., 2002), and nitration of tyrosine residues (Ischiropoulos, 1998). In addition to its effect on protein regulation, NO can also act on DNA (Wink et al., 1991).

NO and the nervous system

In the central nervous system (CNS), NO has been described as a neuromodulator involved in long-term potentiation during learning and memory (O'Dell et al., 1994; Brenman and Bredt, 1996; Son et al., 1996). Recent studies report that presynaptic plasticity induced during activity-dependent synaptogenesis is regulated by NO (Nikonenko et al., 2003; Zhang et al., 2005). NO has also been suggested to play an important role in the formation of retinal projections during neuronal development *in vivo* (Williams et al., 1994; Cramer et al., 1996; Gibbs and Truman, 1998; Cramer and Sur, 1999; Ernst et al., 1999; Wildemann and Bicker, 1999; Wu et al., 2000). For example, inhibition of nNOS by pharmacological blockers was shown to prevent the

elimination of transient ipsilateral retinotectal projections in the chick visual system, and to affect retinogeniculate projection patterns in ferret (Wu et al., 1994; Cramer and Sur, 1999).

Moreover, NO has been reported to be involved in neuronal differentiation and neurite outgrowth. Neuronal differentiation of NGF-induced PC12 cells is dependent on NO (Peunova and Enikolopov, 1995; Nakaya et al., 2000). However, the role of NO on neurite outgrowth is controversial. Some groups have reported that NO induces growth cone collapse and neurite retraction (Hess et al., 1993; Ernst et al., 1999; Gallo et al., 2002), and others have reported that NO facilitates neurite outgrowth (Hindley et al., 1997; Poluha et al., 1997; Rialas et al., 2000). During the development of the grasshopper embryo, NO, potentially released from epithelial cells facing the basal lamina of the developing antenna, has been shown to regulate axonal pathfinding of antennal pioneer neurons (Seidel and Bicker, 2000; Bicker, 2007). We have shown previously that NO donors applied to identified *Helisoma* B5 neurons grown *in vitro* results in an increase in filopodial length and a concomitant decrease in neurite outgrowth (Van Wagenen and Rehder, 1999; Trimm and Rehder, 2004; Welshhans and Rehder, 2005), suggesting that some of the developmental effects of NO described in the literature might result from NO acting on neuronal growth cones. A recent study, in which NO donors were applied focally to B5 growth cones, indeed confirmed that NO regulates filopodial motility locally at the growth cone and that the NO signaling pathway included soluble guanylyl cyclase (sGC), protein kinase G (PKG), cyclic adenosine diphosphate ribose (cADPR) and intracellular calcium release from ryanodine sensitive stores (Welshhans and Rehder, 2005; Welshhans and Rehder, 2007). In addition, a follow up study demonstrated that NO release from a single cell has a physiological effect on growth cone dynamics of neighboring neurons *in vitro* (Tornieri and Rehder, 2007).

NO has also been suggested to have pathological effects in neurodegenerative diseases and in CNS injury (Ruan et al., 1995; Beckman et al., 2001; Tabner et al., 2002). Recent studies have highlighted a controversy in the role of NO in neuronal regeneration. Upregulation of NOS expression has been associated with the degeneration of spinal motoneurons (Wu and Scott, 1993; Wu et al., 1994; Wu, 1996) and with the death of Purkinje neurons (Chen and Aston-Jones, 1994) following injury. However, upregulation of NOS expression has also been correlated with axonal regeneration after injury of cranial motoneurons (Yu, 1994). A regenerative delay due to a decelerated Wallerian degeneration has been reported in NOS knockout mice (Keilhoff et al., 2002). Recently, NO has also been demonstrated to promote axonal regeneration following injury in locust embryos (Stern and Bicker, 2008). Furthermore, iNOS and nNOS have been reported to have a neuroprotective role in the olfactory neuroepithelium (Min et al., 2003), and peroxynitrite has been suggested to be involved in cellular survival at low concentrations (Bolanos et al., 2004). The difference in cell types and species used in the studies mentioned above may explain this discrepancy on the role of NO following CNS injury.

***Helisoma trivolvis* as a Model System to Study Neuronal Pathfinding during Development and Regeneration Following Injury**

Transplantation of embryonic neuronal tissues (Low et al., 1982), solid grafts (Lindvall et al., 1990), and neuronally derived cells (Baron-Van Evercooren, 1994) has been used with limited success in mammalian models to enhance neuronal regeneration beyond the glial scar. Because regeneration occurs in the CNS of *Helisoma trivolvis*, it is a well-established model system to study neuronal regeneration following injury *in vivo* (Murphy and Kater, 1980; Berdan et al.,

1990; Murrain et al., 1990; Berdan and Easaw, 1992) and *in vitro* (Rehder et al., 1992; Geddis and Rehder, 2003; Geddis and Rehder, 2003). *Helisoma* has a simple, well characterized nervous system, and the large size (up to 100 μm) and consistent position of the neuronal cell bodies in its ganglia permits (i) the injection of tracer dyes into identified neurons to assess changes in morphology during regeneration (Murphy and Kater, 1980; Berdan et al., 1990; Berdan and Easaw, 1992), and (ii) isolation of identified neurons into primary culture. Identification of individual neurons in different animals by their morphology, color, location and size allows for elimination of variability due to a heterogeneous population of neurons. In addition, the ability to maintain organ and cell culture allows for complete control over the environment during the experiment. To address the specific aims of my dissertation, I performed experiments using primary cell culture of two identified neurons (neurons B5 and B19) located in the buccal ganglion, which controls the feeding behavior of the snail.

Morphology and characteristic of B5 and B19 buccal neurons

In vivo one single axon of the B5 neuron exits the buccal ganglion through the esophageal nerve trunk and branches near the salivary gland (i) to innervate the esophagus via the gastric nerve and (ii) to innervate the buccal mass via the dorsobuccal nerve trunk (Berdan RC, 1989; Berdan et al., 1990). B5 neurons are cholinergic and nitergic neurons, in which electrical activity is modulated by glutamate (Haydon et al., 1990; Zoran et al., 1990; Van Wagenen and Rehder, 2001; Scannell et al., 2008). The B19 neuron innervates the supralateral radular tensor muscle of the buccal mass (Berdan RC, 1989; Zoran et al., 1990). B19 neurons are cholinergic neurons, in which glutamate affects electrical activity, and serotonin and dopamine decrease neurite outgrowth (Haydon et al., 1984; Cohan et al., 1987; McCobb et al., 1988; Haydon et al., 1990;

MurRAIN et al., 1990; Zoran et al., 1990; Davenport et al., 1993; Scannell et al., 2008). The fact that B19 neurons do not express nNOS and sGC, as opposed to B5 neurons, makes them a good control in experiments investigating the role of NO (Van Wagenen and Rehder, 2001; Tornieri and Rehder, 2007).

Helisoma growth cones

The large size of growth cones from neurons grown in culture (up to 50 μ M) makes *Helisoma trivolvis* a great system to study growth cone dynamics. Neurons cultured in defined medium (without growth factors) form short neurites with large non-motile growth cones (Williams and Cohan, 1994), suggesting that growth cone formation is an intrinsic property, whereas neurite advance requires growth factors. These large growth cones permit the investigation of cytoskeletal changes during growth cone dynamics (Cohan et al., 2001; Zhou and Cohan, 2001). On the other hand, culturing neurons in conditioned medium (containing growth factors) induces the formation of extended neurites tipped with motile growth cones (Williams and Cohan, 1994), suggesting that neurite extension requires growth factors. The morphology and motility of B5 and B19 growth cones differ, with B5 growth cones having shorter and more filopodia than B19 growth cones (Haydon et al., 1985). In terms of outgrowth, B5 growth cones advance faster than B19 ones (Haydon et al., 1985). To summarize, the simplicity of its nervous system, the large size and accessibility of its neurons, and the fact that its CNS can regenerate leads to the use of *H. trivolvis* as a favorable system to investigate neuronal pathfinding.

Chapter 2 : Control of Neurite Outgrowth and Growth Cone Motility by Phosphatidylinositol-3-Kinase

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Abstract

Phosphatidylinositol-3-kinase (PI-3K) has been reported to affect neurite outgrowth both *in vivo* and *in vitro*. Here we investigated the signaling pathways by which PI-3K affects neurite outgrowth and growth cone motility in identified snail neurons *in vitro*. Inhibition of PI-3K with wortmannin (2 μ M) or LY 294002 (25 μ M) resulted in a significant elongation of filopodia and in a slow-down of neurite outgrowth. Experiments using cytochalasin and blebbistatin, drugs that interfere with actin polymerization and myosin II activity, respectively, demonstrated that filopodial elongation resulting from PI-3K inhibition was dependent on actin polymerization. Inhibition of strategic kinases located downstream of PI-3K, such as Akt, ROCK and MEK, also caused significant filopodial elongation and a slow-down in neurite outgrowth. Another growth cone parameter, filopodial number, was not affected by inhibition of PI-3K, Akt, ROCK, or MEK. A detailed study of growth cone behavior showed that the filopodial elongation induced by inhibiting PI-3K, Akt, ROCK and MEK was achieved by increasing two motility parameters: the rate with which filopodia extend (extension rate) and the time that filopodia spend elongating. Whereas the inhibition of ROCK or Akt (both activated by the lipid kinase activity of PI-3K) and MEK (activated by the protein kinase activity of PI-3K) had additive effects, simultaneous inhibition of Akt and ROCK showed no additive effect. We further demonstrate that the effects on filopodial dynamics investigated were calcium-independent. Taken together, our results suggest that inhibition of PI-3K signaling results in filopodial elongation and a slow-down of neurite advance, reminiscent of growth cone searching behavior.

Introduction

During development growth cones play an important role in guiding neuronal processes to their targets. Growth cones function by integrating intracellular signals that are elicited by the binding of extracellular cues to membrane-bound or intracellularly located receptors, and by translating these signals into a coordinated motor output that results in directed neurite advance. The filopodia on growth cones undergo continuous cycles of extension and retraction, thereby placing their receptors at varying distances from the growth cone proper and resulting in changes in the 'sensory radius' of the growth cone. Filopodial length and neurite outgrowth are under the control of extracellular cues, and several treatments, mainly acting through an increase in the intracellular calcium concentration have been demonstrated to cause a transient increase in filopodial length (Davenport and Kater, 1992; Rehder and Kater, 1992; Cheng et al., 2002) and a decrease in neurite extension (Cohan et al., 1987; Mattson and Kater, 1987; Lankford and Letourneau, 1989; Kater and Mills, 1991; Neely, 1993).

Phosphatidylinositol-3-kinase (PI-3K) has been implicated in a variety of cellular activities, including cell survival, mitogenesis, and differentiation (Krasilnikov, 2000; Rodgers and Theibert, 2002). PI-3K is activated by binding to receptor- and non-receptor tyrosine kinases, as well as by G-protein coupled receptors, and its activation is known to affect multiple targets (Raffioni and Bradshaw, 1992; Soltoff et al., 1992; Soltoff et al., 1992; Duronio et al., 1998; Wymann and Pirola, 1998; Virdee et al., 1999; Krasilnikov, 2000; Cantrell, 2001). In addition to its involvement in mediating cell survival, PI-3K has been reported to stimulate neurite outgrowth in PC12 cells (Kita et al., 1998), to mediate ephrin A5 and slit-2 induced growth cone collapse in chick retinal ganglion cells (Wong et al., 2004), and to be required for the NCAM-mediated neurite outgrowth of primary neurons (Ditlevsen et al., 2003). In addition,

PI-3K has been shown to play a role in lamellipodial formation of PC12 cells (Posern et al., 2000) and in the regulation of neuronal polarity (Shi et al., 2003; Menager et al., 2004). These effects are likely mediated by changes at the level of the neuronal cytoskeleton. The mechanism by which PI-3K affects neurite outgrowth is presently unclear, but prominent targets downstream of PI-3K include Akt (PKB), regulators of Rho-GTPase family members, and the RAS/RAF/ERK pathway (Corvera and Czech, 1998; Duronio et al., 1998; Vanhaesebroeck and Waterfield, 1999; Bishop and Hall, 2000; Krasilnikov, 2000; Cantrell, 2001). To determine whether the effect of PI-3K activity on neurite outgrowth might be explained by PI-3K affecting growth cone motility through its effects on the neuronal cytoskeleton, we here studied several key targets within the larger PI-3K signaling cascade to investigate the signaling pathway by which PI-3K affects the actin cytoskeleton of neuronal growth cones.

PI-3K has a double-enzymatic activity, that of a lipid kinase and a protein kinase (Dhand et al., 1994; Krasilnikov, 2000). The protein kinase activity regulates the PKC, Ras/ERK, and Rac/JNKK/JNK signaling pathways, whereas the lipid kinase activity controls the Akt anti-apoptotic signaling pathway and the activity of Rho kinase (ROCK) (Krasilnikov, 2000; Cantley, 2002). By using two inhibitors of PI-3K, wortmannin and LY 294002, we show that PI-3K activity is involved in regulating both neurite outgrowth and filopodial dynamics. We further show that this regulation involved members of the lipid kinase (ROCK or Akt) and the protein kinase (MEK) pathways of PI-3K signaling. Fast time course studies of filopodial dynamics showed that the rapid filopodial elongation seen after different treatments was achieved mainly by changes in two parameters of filopodial motility: increases in the extension rate and in the time that individual filopodia spend extending. By employing drugs that interfere with actin polymerization and myosin II activity, we further provide evidence that filopodial elongation

seen after inhibition of PI-3K is mainly due to an increase in actin polymerization at the barbed end, and not through inhibition of retrograde actin flow. Because filopodial elongation and a decrease in the rate of neurite outgrowth seen after inhibition of PI-3K in this study are similar to growth cone behavior observed at “decision points” during pathfinding *in vivo* (Taghert et al., 1982; Tosney and Landmesser, 1985; Holt, 1989), these data suggest that signaling through PI-3K could be of critical importance for growth cone motility, and that a lack of stimulation or inhibition of the PI-3K pathway could result in slow-down and search behavior in advancing neurites.

Methods and Materials

Neuronal culture and image acquisition

Identified B5 neurons were dissected from *Helisoma trivolvis* buccal ganglia and plated for neuronal culture. Neurons used for filopodial experiments were plated on poly-L-lysine (hydrobromide, MW 70,000-150,000, 0.25mg/ml; Sigma, St. Louis, MO) coated glass cover slips that were glued to the bottom of 35 mm cell culture dishes (Falcon 1008). Neurons used for outgrowth experiments were plated onto either the same glass dishes described above, or onto poly-L-lysine coated plastic cell culture dishes as described in Trimm and Rehder (2004). Cells were kept at room temperature and used 24-48 hours after plating. Neurons were grown in conditioned medium (CM) prepared by incubating two snail brains per milliliter in defined medium (DM) for 3-4 days. DM was produced by dilution of Leibowitz L-15 medium (Gibco #41300, Grand Island, NY) with distilled water to obtain the appropriate ionic composition and contained final concentrations of: 46 mM NaCl, 1.8 mM KCl, 1.5 mM MgCl₂, 4.1 mM CaCl₂, 10 mM HEPES, 50 µg gentamycin/ml, 0.15 mg glutamate/ml in distilled water (pH=7.3). Growth

cones were viewed through 100x oil immersion objectives on either an Axiovert (Carl Zeiss, Thornwood, NY) or a Sedival (aus Jena, Germany) microscope. Neurite outgrowth was imaged through a 40x objective on a Sedival microscope. Images were captured with a cooled CCD camera (CH250, Photometrics, Tucson AZ) or a regular CCD camera (Model: C-72, MIT Dage, Michigan City, IN). Images were digitized on a frame grabber (Scion LG-3, Scanalytics, Fairfax, VA) and analyzed with “Scion Image” software (Scion Corporation; Frederick, Maryland) on a Power Macintosh (Apple Computer, Cupertino, CA) or a Compaq Presario (Hewlett-Packard Company, Palo Alto, CA).

Pharmacological agents

LY 294002 (PI-3K inhibitor), PD 98059 (MAP kinase kinase inhibitor), Akt inhibitor, blebbistatin (nonmuscle myosin II ATPase inhibitor), and cytochalasin B (actin polymerization inhibitor) were dissolved in DMSO to obtain stock concentrations of 20, 50, 45, 50 mM and 60 μ M respectively. Wortmannin, another PI-3K inhibitor, was dissolved in methanol to a stock concentration of 1 mM. The Rho-kinase inhibitor Y27632 was dissolved in water to a stock concentration of 10 mM. All agents were purchased from Calbiochem (San Diego, CA).

Design and analysis of filopodial experiments

Observation of growth cone behavior, specifically, changes in filopodial length and filopodial number, was performed at defined times before and after the addition of pharmacological agents, which were added to the dish through a complete medium exchange. Immediately before each experiment, drugs were premixed in 2 mL of L-15 medium to obtain

final concentrations. Control experiments were performed for each of the solvents used in the reconstitution of pharmacological agents and, in each case, the final solvent concentration did not exceed 0.2%. At these concentrations, the solvents had no significant effect on filopodia. Images were captured before (-5 and 0 min) and at defined times after (2, 5, 10, 15, 20, and 30 minutes) a complete medium exchange, which introduced the experimental condition.

Digitized images of growth cones were analyzed by tracking all filopodia from image to image in previously defined intervals, and by measuring the length of filopodia from their base to their tip. The filopodial base was defined as the transition point where the lamellipodial peripheral domain meets the organelle rich central domain of the growth cone. Filopodial length was measured from phase contrast images using the software package “Scion Image.” All filopodia on a growth cone were measured and those values were averaged to result in a measure of ‘mean filopodial length’ for each growth cone. These values were expressed as a percentage change normalized to the time point $t = 0$ just before any drug treatment to compensate for the fact that individual growth cones varied in size and filopodial length.

Fast time-course experiments were performed on single growth cones by tracking individual filopodia from image to image as previously described (Van Wagenen et al., 1999). Images were acquired every 30 seconds during 7 minutes before treatment (Pre) and 10 minutes after treatment (Post). This experimental design allows for determination of the parameters of filopodia dynamics, such as the time a filopodium spends extending, retracting, or being stable. Moreover, the extension and retraction rates of individual filopodia can be measured with this approach. Filopodia were classified as extending or retracting if their individual filopodial length increased or decreased by $0.4 \mu\text{m}$ in 30 seconds. Filopodia that did not meet this criterion were considered as stable. Filopodia extension and retraction rates were expressed in $\mu\text{m}/30 \text{ sec}$.

Design and analysis of neurite outgrowth experiments

Images of neurons were taken at 20 minutes intervals for a total of 2 hours per experiment as previously described (Trimm and Rehder, 2004). Neurite outgrowth was determined by measuring neurite length starting from a fixed point in the dish (e.g. soma) and measuring the distance to the growth cone's central domain using the software package "Scion Image." Neurites that advanced less than 6 μm during the control period (one hour before treatment) or that fasciculated with other neurites during the experiment were excluded from analysis. Outgrowth during the first hour of treatment following drug addition was measured by subtracting neurite length at $t = 0$ from $t = 60$. Outgrowth during the second hour of treatment following drug addition was measured by subtracting neurite length at $t = 60$ from $t = 120$. In order to introduce the experimental condition, 0.2 mL of conditioned medium was removed from the dish, a drug was premixed in this volume, and the 0.2 mL was subsequently added back to the dish to obtain the final concentration. Solvents used in the reconstitution of pharmacological agents were tested by themselves in control experiments and had no significant effect on neurite outgrowth.

Calcium imaging

Calcium imaging was performed as described previously (Rehder and Cheng, 1998; Van Wagenen and Rehder, 1999). Briefly, *Helisoma* B5 neurons were pressure injected (Picospritzer; General Valve Corp., Fairfield, NJ) with the calcium indicator dye, Fura-2 pentapotassium salt (10 mM in water; Molecular Probes, Eugene, OR). Cells were used 20-30 minutes after injection in order to allow for the calcium indicator to diffuse. Fura-2 was excited at two excitation wavelengths

(340 ± 10 nm and 380 ± 10 nm) and after calibration, the ratio of the fluorescent images captured was converted into an estimated $[Ca^{2+}]_i$ according to Grynkiewicz et al. (1985).

Actin labeling and analysis of actin fluorescence

Growth cones were fixed and stained according to Cohan et al. (2001) with the variation that rhodamine phalloidin was used (Molecular Probes) at a concentration of $0.165 \mu\text{M}$. Growth cones were viewed through a 100x oil immersion objective on a Zeiss Axiovert inverted microscope equipped with a rhodamine filter set (Omega Optical, Brattleboro, VT). Images were acquired using a cooled Photometrics CCD camera, and stored on a Macintosh computer. A camera exposure time of 200 ms was used for all images. Image intensity was analyzed with Scion Image software (Scion Corporation) and two measurements were taken to quantify f-actin content in growth cones. To calculate the mean fluorescent intensity of f-actin in the growth cone's p-region, a line was drawn outlining the lamellipodial region and the measured fluorescence averaged. The fluorescent intensity of actin bundles forming the core of filopodia was analyzed according to Cohan et al. (2001). Briefly, a line was drawn across individual actin bundles within the middle of the lamellipodium. The peak intensities for each bundle (the average of 3 pixels for each bundle) were determined and this procedure repeated for at least 5 filopodia per growth cone, except when that was impossible due to loss of filopodia (condition with 500 nM Cytochalasin B). These values were then expressed as an average peak intensity for each growth cone. Staining was done in parallel for different experimental conditions to minimize differences in staining intensity between dishes.

Statistical analysis

For filopodial experiments, significance between conditions was determined by performing an analysis of variance (1-way ANOVA or ANOVA with time as repeated measures), and with a *t*-test for dependent and independent samples, with SuperANOVA (Abacus Concepts, Berkeley, CA). Fisher's protected least significant difference (PLSD) test was used for post-hoc tests of statistical significance. For neurite outgrowth, fast time-course and actin labeling experiments, significance between conditions was determined by performing a Student's *t*-test (for unpaired or paired samples) with Excel (Microsoft; Redmond, WA). Significant differences are indicated as: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Results

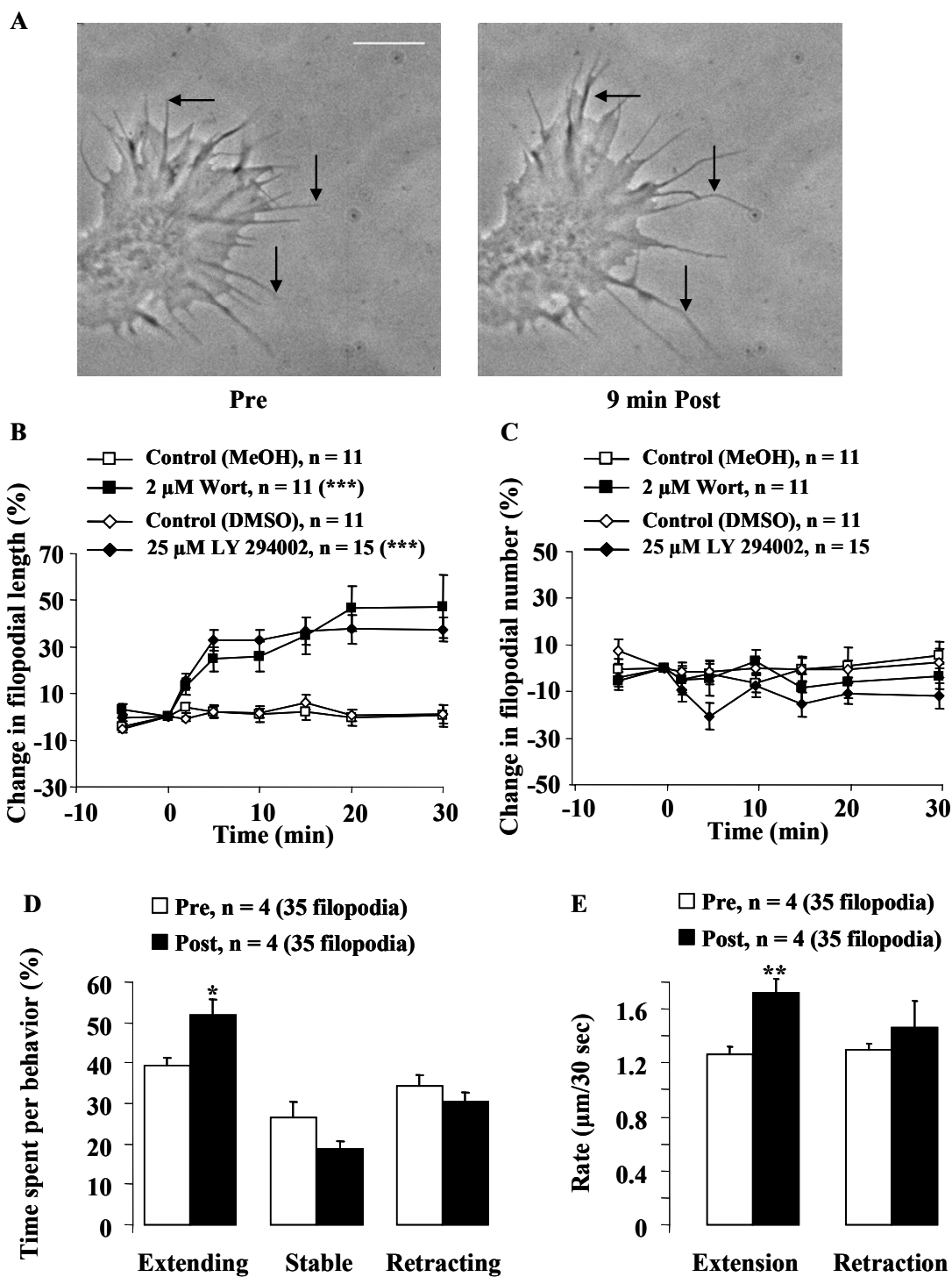
PI-3K inhibition induces filopodial elongation and a slow-down in the rate of neurite advance

To investigate the effect of PI-3K activity on growth cone motility, B5 neurons were treated with two specific PI-3K inhibitors, wortmannin and LY 294002. Treatment with LY 294002 (25 μ M) resulted in filopodial elongation as early as five minutes after addition of the drug, with maximal overall filopodial elongation of $37.6 \pm 6.1\%$ reached after 20 min (Fig. 2.1A and 1B) (see Supplemental time-lapse movie). This elongation effect was highly significantly when compared to the control condition ($p \leq 0.001$), in which solvent alone (DMSO 0.125%) was applied. Interestingly, the number of filopodia on a given growth cone, another parameter of growth cone morphology, was not affected by LY 294002 ($p = 0.70$) (Fig. 2.1C). To confirm independently the effect of PI-3K inhibition on growth cone morphology, neurons were treated with a second PI-3K inhibitor, wortmannin (Wort, 2 μ M). At this concentration, the overall filopodial length increased significantly by $46.6 \pm 9.6\%$ after 20 min ($p \leq 0.001$) compared to the

Figure 2.1: Inhibition of PI-3K results in an increase in filopodial length by increasing the time filopodia spent extending and the filopodial extension rate, but has no effect on filopodial number.

(A) Phase-contrast images of a representative B5 growth cones 5 minutes before (pre) and 9 minutes after treatment with the PI-3K inhibitor, LY 294002 (25 μ M). Note that a majority of filopodia elongate (representative filopodia are indicated with an arrow). Scale bar = 12 μ m. (see Supplemental time-lapse movie of this growth cone). (B) Treatment of B5 growth cones with LY 294002 (25 μ M) caused a significant increase in overall filopodial length as early as 5 min after addition of the drug. Exposure of growth cones to DMSO (0.125%), the solvent in which LY 294002 was dissolved, had no effect on filopodial length ($p = 0.70$). The effect of LY 294002 was significantly different from the control condition ($p \leq 0.001$), as indicated in *parentheses* behind the treatment group. PI-3K inhibition with a second PI-3K inhibitor, wortmannin (2 μ M), also caused a rapid significant increase in overall filopodial length when compared to its methanol control (0.2%) ($p \leq 0.001$). (C) The number of filopodia on a given growth cone was not affected by either LY 294002 (25 μ M) or wortmannin (2 μ M) treatment and there also was no significant difference between the two drug treatments ($p = 0.644$). (D) Inhibition of PI-3K with 25 μ M LY 294002 resulted in a significant increase in the time filopodia spent extending in the first five minutes following drug addition (Post) in comparison to 7 min prior to drug addition (Pre). The time spent stable and retracting were not significantly affected by LY 294002 (25 μ M) treatment ($p = 0.12$ and $p = 0.36$ respectively). (E) Treatment with 25 μ M LY 294002 caused a significant increase in the filopodial extension rate in comparison to its control (Pre, 7 min prior to drug addition) ($p \leq 0.01$). Filopodial retraction rate was not affected by LY 294002 (25 μ M) treatment ($p = 0.45$).

For all experiments the number of growth cone studied per condition is given as 'n' and listed behind each experimental condition. Statistical significance between conditions is symbolized by *asterisks* as follows: * $P \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.



solvent control (Fig. 2.1B). Note that the filopodial elongation induced by LY 294002 and wortmannin were not significantly different ($p = 0.98$), suggesting that the two inhibitors blocked PI-3K to a similar degree when used at these concentrations. Consistent with the observation made with the other PI-3K inhibitor (LY 294002), treatment with wortmannin also had no significant effect on filopodial number ($p = 0.64$) (Fig. 2.1C).

Growth cone filopodia frequently switch between episodes of extension and retraction within seconds. Whereas the measurements above captured the average length and number of filopodia at fixed time points that were separated by several minutes, we next wanted to investigate the dynamic process of filopodial elongation in more detail. Filopodial length changes can be explained by underlying changes in dynamic filopodial parameters, such as the amount of time individual filopodia spend extending, retracting or not moving (stationary), as well as the rates at which filopodia extend and retract. To investigate which parameters of filopodial dynamics were affected to explain the filopodial elongation observed after inhibition of PI-3K, individual filopodia on single growth cones were tracked every 30 sec during a 7 min pretreatment period (Pre), and during the initial 4-5 minutes after treatment that included the phase of rapid filopodial elongation (Post). Treatment with LY 294002 significantly increased the time that filopodia spent extending from 39.5 ± 1.6 % before treatment to 52.0 ± 3.6 % after treatment ($p \leq 0.05$) (Fig. 2.1D). The times filopodia were stationary and spent retracting were not affected ($p = 0.12$ and $p = 0.36$ respectively) (Fig. 2.1D). The filopodial extension rate was significantly increased from 1.26 ± 0.06 $\mu\text{m}/30$ sec before treatment (Pre) to 1.72 ± 0.1 $\mu\text{m}/30$ sec after treatment with LY 294002 ($p \leq 0.01$) (Fig. 2.1E). Treatment with the PI-3K inhibitor did not affect the filopodial retraction rate ($p = 0.45$) (Fig. 2.1E). These results suggested that the overall filopodial elongation seen in response to PI-3K inhibition

resulted from the combination of an increase in the extension rate and the time that filopodia spent extending.

We next investigated whether PI-3K inhibition would also affect neurite outgrowth. Indeed, treatment with LY 294002 significantly reduced the neurite outgrowth rate from $18.2 \pm 1.7 \mu\text{m}$ in the hour prior to treatment to $8.5 \pm 2.6 \mu\text{m}$ ($p \leq 0.001$) in the first hour after treatment (Fig. 2.2). A further significant decrease in outgrowth to $3.2 \pm 1.7 \mu\text{m}$ resulted in the second hour of treatment. Taken together, these results suggested that a decreased stimulation, or inhibition of the PI-3K pathway, could serve as an acute slow-down and search signal for migrating growth cones.

MEK, Akt and ROCK regulate filopodial dynamics and neurite outgrowth

To investigate the pathway by which PI-3K might regulate growth cone motility, we pharmacologically inhibited major targets downstream of PI-3K with the assumption that their inhibition should mimic the effect of PI-3K inhibition and result in filopodial elongation and/or a slow down in neurite outgrowth. PI-3K has a double enzymatic activity, acting as a protein kinase and a lipid kinase (Krasilnikov, 2000; Cantley, 2002) (Fig. 2.3). Therefore, we wanted to investigate which of the two activities might be involved in controlling filopodial dynamics and neurite outgrowth.

We first inhibited Akt and ROCK, which are activated by the lipid kinase activity of PI-3K. Akt was blocked with the Akt inhibitor (80 μM) and ROCK was inhibited with Y27632 (10 μM) (Fig. 2.3). Treatment with Akt inhibitor and Y27632 increased filopodial length by $28.4 \pm 2.4\%$ and $23.6 \pm 2.4\%$ respectively in comparison to the control condition ($p \leq 0.001$) (Fig.

Figure 2.2: Inhibition of PI-3K results in a slow-down of neurite outgrowth.

Inhibition of PI-3K with 25 μ M LY 294002 resulted in a significant decrease in neurite outgrowth in the first hour following drug addition ($p \leq 0.001$). A further significant decrease was seen in the second hour following drug addition ($p \leq 0.001$ as compared to the hour prior to drug addition). Note that addition of the solvent control (DMSO) by itself had no effect on neurite outgrowth.

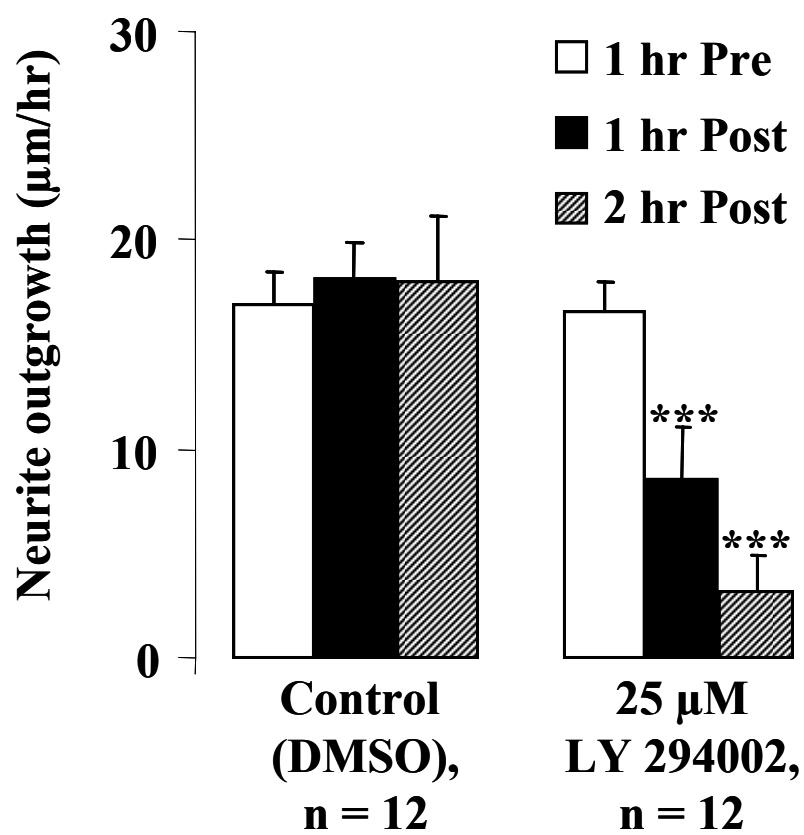


Figure 2.3: Investigated signaling pathways by which PI-3K may regulate growth cone dynamics.

PI-3K is activated by multiple effectors. The PI-3K signaling pathway is bifurcated and has two branches, resulting from its lipid- and protein kinase activities. Through its lipid kinase activity, PI-3K phosphorylates phosphatidylinositol (PI) and its phosphorylated derivatives. Then phosphoinositides activate the RhoA/ROCK pathway, and the phosphoinositide-dependent kinase (PDK), which in turn activates Akt. Through its protein kinase activity (in gray), PI-3K binds directly to Ras and Rac-Cdc42, leading to the activation of MEK. Kinases investigated in this study are in bold typeface and underlined. Inhibitors are indicated in *italics*.

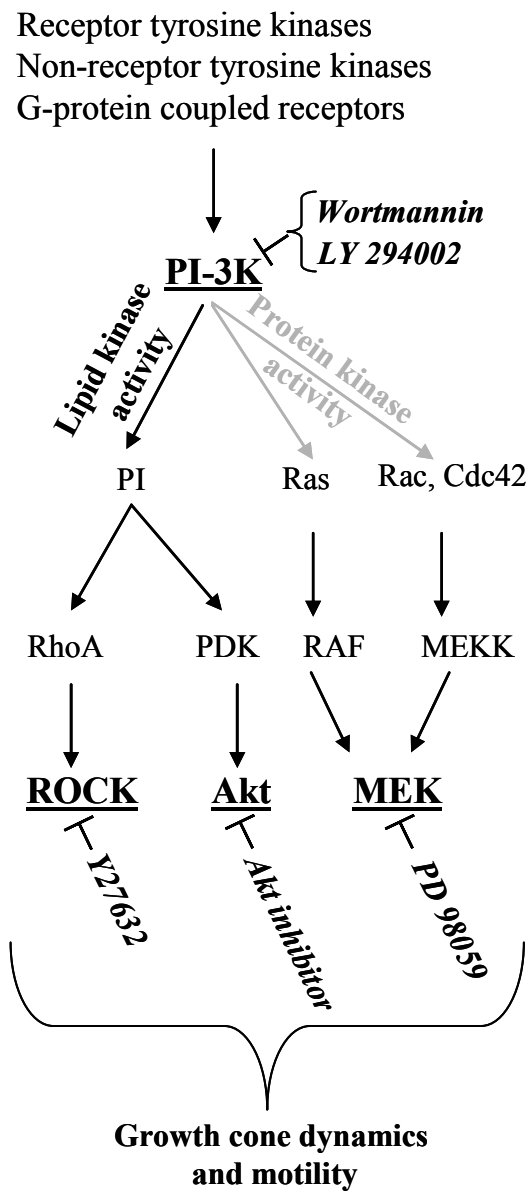
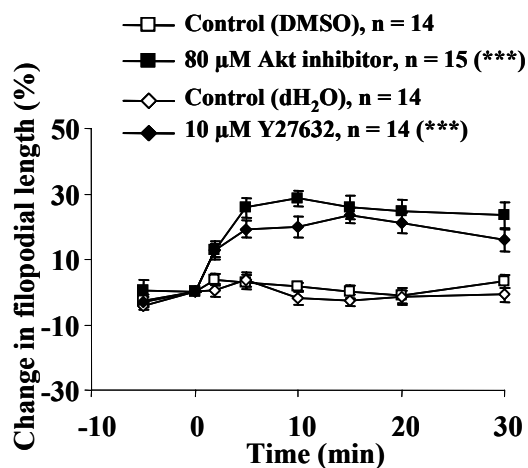


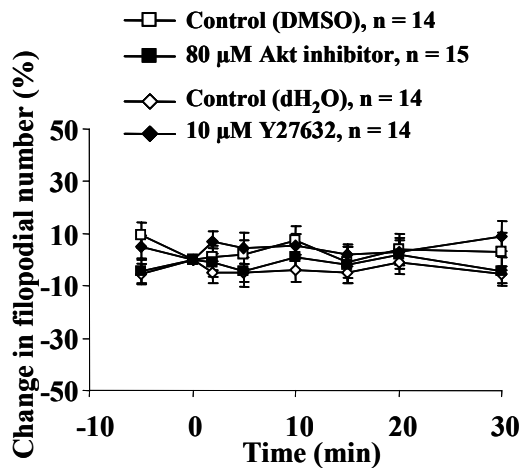
Figure 2.4: Regulation of filopodial dynamics by Akt and ROCK. (A-F)

The involvement of the lipid kinase activity of PI-3K was tested by strategically inhibiting key kinases downstream of PI-3K activity (see Fig. 2.3). **(A, B)** Two targets of the PI-3K lipid activity, namely Akt and ROCK, were inhibited with Akt inhibitor, and with Y27632 respectively. **(A)** Both Akt inhibitor (80 μ M) and ROCK inhibitor, Y27632 (10 μ M), significantly increased overall filopodial length in comparison to their respective control conditions, 0.175% DMSO and 0.1% water (dH₂O) (both $p \leq 0.001$). **(B)** Filopodial number was not significantly altered by the inhibition of Akt with Akt inhibitor (80 μ M) ($p = 0.44$) or by the inhibition of ROCK with Y27632 (10 μ M) ($p = 0.07$) when compared to controls. **(C)** Inhibition of Akt with Akt inhibitor (80 μ M) resulted in a significant increase in the time filopodia spent extending in the first five minutes following drug addition (Post) compared to the 7 min control time window prior to drug addition (Pre) ($p \leq 0.05$). The times spent stable and retracting were not significantly affected by Akt inhibitor (80 μ M) treatment ($p = 0.05$ and $p = 0.85$ respectively). **(D)** Treatment with 80 μ M Akt inhibitor caused a significant increase in filopodial extension rate compared to control (Pre, 7 min prior to drug addition) ($p \leq 0.05$). The filopodial retraction rate was not affected by Akt inhibitor (80 μ M) ($p = 0.24$). **(E)** Inhibition of ROCK with Y27632 (10 μ M) resulted in a significant increase in the time filopodia spent extending ($p \leq 0.01$), as well as a significant decrease in the time filopodia spent being stable ($p \leq 0.05$). The time spent retracting was not significantly affected by Y27632 (10 μ M) treatment ($p = 0.09$). **(F)** Treatment with 10 μ M Y27632 caused a significant increase in filopodial extension and retraction rates compared to controls (Pre, 7 min prior to drug addition) ($p \leq 0.01$ and $p \leq 0.05$ respectively).

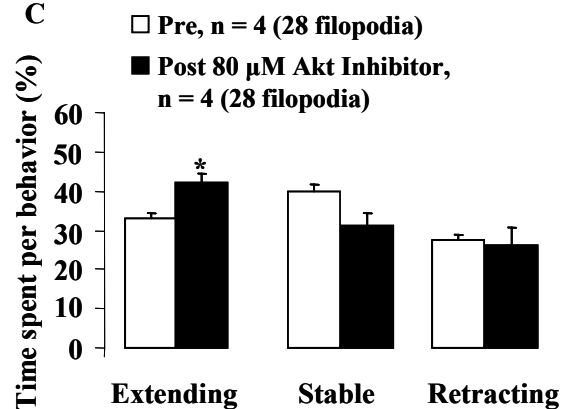
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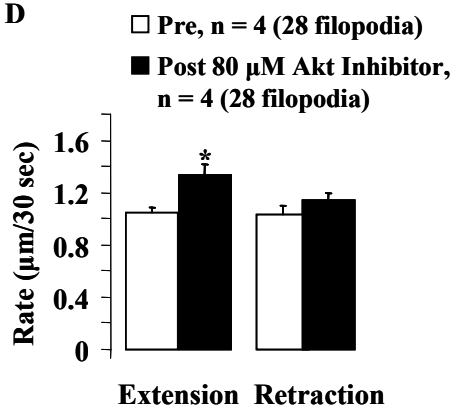
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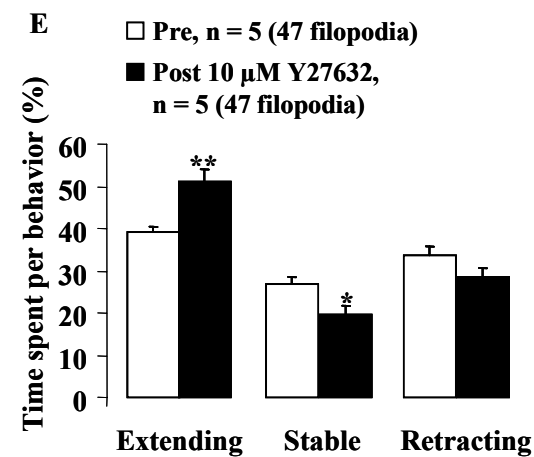
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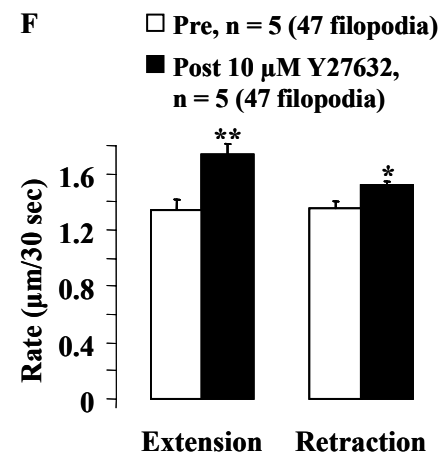
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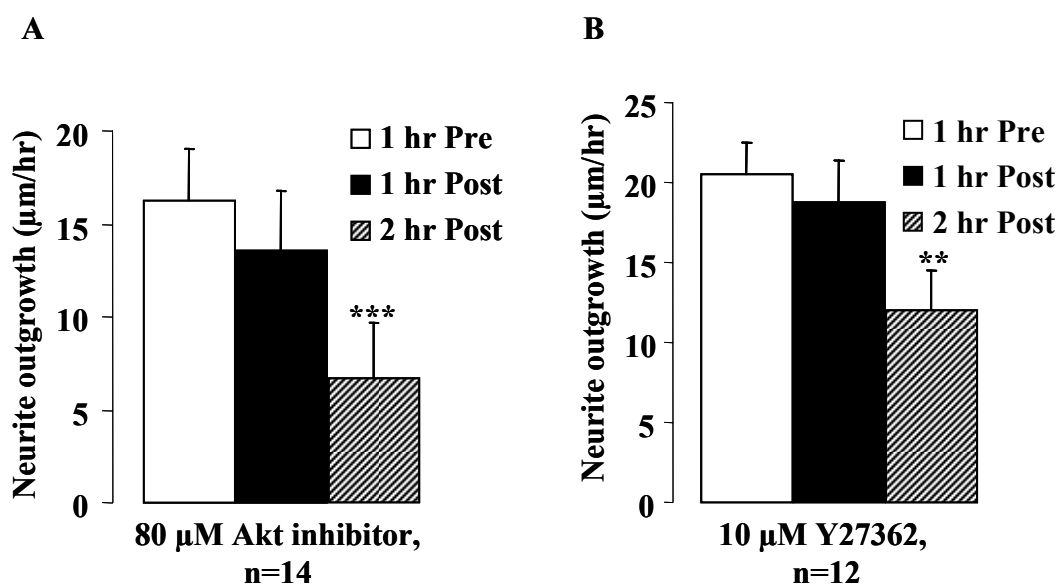
2.4A). Filopodial number was not affected by these treatments ($p = 0.44$ and $p = 0.07$ respectively) (Fig. 2.4B).

Next, we investigated which parameters of filopodial dynamics were affected by inhibiting targets of the lipid kinase pathways of PI-3K. Therefore, we monitored filopodial dynamics in fast time-course experiments as described earlier. Treatment with Akt inhibitor increased significantly the time filopodia spent extending from $32.9 \pm 1.6\%$ before treatment (Pre) to $42.3 \pm 2\%$ after treatment (Post) ($p \leq 0.05$) (Fig. 2.4c). The times filopodia were stationary and spent retracting were not affected by Akt inhibitor ($p = 0.05$ and $p = 0.85$ respectively) (Fig. 2.4C). The filopodial extension rate was also significantly increased from $1.04 \pm 0.04 \mu\text{m}/30 \text{ sec}$ before treatment (Pre) to $1.33 \pm 0.08 \mu\text{m}/30 \text{ sec}$ after treatment ($p \leq 0.05$; Fig. 2.4D). The ROCK inhibitor, Y27632 induced a significant increase in the time filopodia spent extending from $39.3 \pm 1.2\%$ before treatment (Pre) to $51.2 \pm 3\%$ after treatment (Post) ($p \leq 0.01$; Fig. 2.4E). The time filopodia were stationary was significantly decreased from $26.9 \pm 1.4\%$ to $19.8 \pm 2.1\%$ ($p \leq 0.05$) as compared to controls (Pre), whereas the time filopodia spent retracting was not affected ($p = 0.09$) (Fig. 2.4E). Inhibition of ROCK also increased both the extension and the retraction rates from $1.34 \pm 0.07 \mu\text{m} / 30 \text{ sec}$ to $1.73 \pm 0.08 \mu\text{m} / 30 \text{ sec}$, and from $1.35 \pm 0.05 \mu\text{m} / 30 \text{ sec}$ to $1.51 \pm 0.03 \mu\text{m} / 30 \text{ sec}$ respectively ($p \leq 0.01$ and $p \leq 0.05$) (Fig. 2.4F).

The effect of inhibition of Akt and ROCK on neurite outgrowth was studied next. When Akt was inhibited, neurite outgrowth was not affected in the first hour following drug addition ($16.2 \pm 2.8 \mu\text{m}$ in the hour prior to drug addition and $13.6 \pm 3.2 \mu\text{m}$ in first hour following drug addition; Fig. 2.5A). However, in the second hour following drug addition, neurite outgrowth was significantly slowed to $6.7 \pm 3.0 \mu\text{m}$ ($p \leq 0.001$ as compared to the hour prior to drug addition). When ROCK was inhibited with Y27362 neurite outgrowth also was not affected in

Figure 2.5: Regulation of neurite outgrowth by Akt and ROCK.

(A) Inhibition of Akt with Akt inhibitor (80 μ M) did not significantly affect neurite outgrowth in the first hour following drug addition. However, neurite outgrowth was slowed significantly in the second hour following drug addition ($p \leq 0.001$). **(B)** Inhibition of ROCK with 10 μ M Y27362 did not significantly affect neurite outgrowth in the first hour after drug addition. However, neurite outgrowth was slowed significantly in the second hour following drug addition (Student's t-test, $**p \leq 0.01$).



the first hour following drug addition ($20.5 \pm 2.1 \mu\text{m}$ in the hour prior to drug addition and $18.8 \pm 2.6 \mu\text{m}$ in first hour following drug addition; Fig. 2.5B). However, in the second hour following drug addition, neurite outgrowth was significantly decreased to $12.0 \pm 2.6 \mu\text{m}$ ($p \leq 0.01$ as compared to the hour prior to drug addition). Taken together, these results are consistent with the idea that the lipid kinase activity of PI-3K is involved in controlling filopodial dynamics and neurite outgrowth through Akt and ROCK. To determine whether the decreases in neurite outgrowth rates observed in the first or second hour after the inhibition of PI-3K, Akt and ROCK were due to an adverse effect of the long-term exposure of the drugs on filopodial dynamics, we measured the dynamic parameters of filopodia after 2 hrs of treatment with LY 294002, Akt inhibitor and Y27632 (Table 2.1).

A comparison of filopodial behaviors (time filopodia spend extending, stationary, or retracting) revealed that there were no significant differences between these filopodial behaviors before and two hours after treatment, with the exception of the Akt inhibitor resulting in filopodia spending more time retracting, and Y 27632 resulting in filopodia spending less time stationary and more time retracting (Table 2.1). Moreover significant long term effects of these drugs on filopodial extension and retraction rates when comparing these parameters before and two hours after treatment. These results suggest that filopodia remained motile during long-term drug treatment and that the decrease in neurite outgrowth rate observed in response to LY 294002, Akt inhibitor and Y27632 treatments likely results from mechanisms other than affecting filopodial dynamics. We next inhibited MAP kinase kinase (MEK), which is activated by the protein kinase activity of PI-3K (Fig. 2.3). After treatment with specific MEK inhibitor PD 98059 ($40 \mu\text{M}$), the filopodial length increased by $23.0 \pm 3.1\%$ (Fig. 2.6A), and this elongation was significant when compared to the control group ($p \leq 0.001$), which showed no

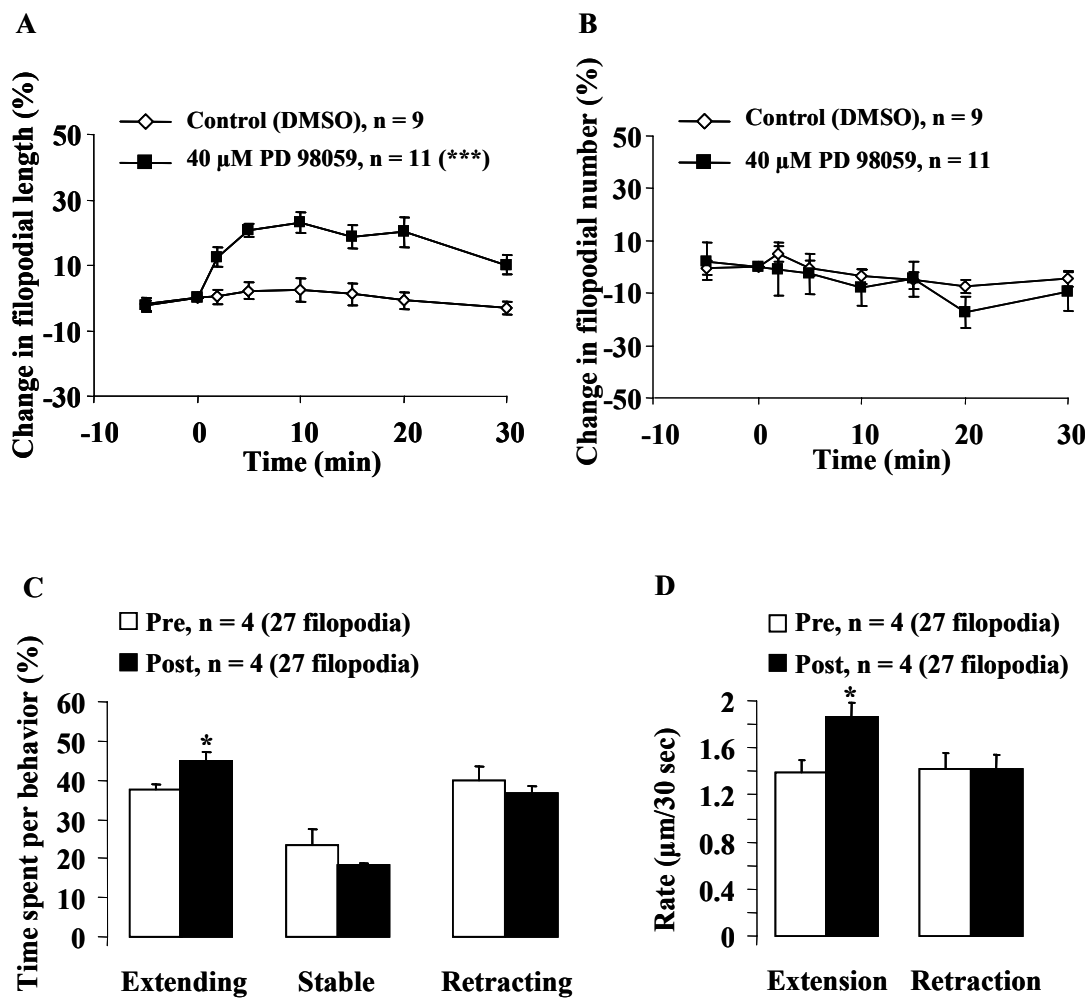
Table 2.1: Comparison of filopodial parameters before and two hours after drug treatments.

* $p \leq 0.05$ compared to control (Pre).

| | PI-3K | Akt | ROCK |
|------------------------|-------------------|-------------------|-------------------|
| | Inhibition | Inhibition | Inhibition |
| % Time spent | | | |
| Extending (pre) | 39.5 ± 1.6 | 32.9 ± 1.6 | 39.3 ± 1.2 |
| Extending (2 hr post) | 42.5 ± 5.0 | 35.6 ± 5.2 | 38.9 ± 2.7 |
| Stable (pre) | 26.4 ± 3.8 | 39.8 ± 2.0 | 26.9 ± 1.4 |
| Stable (2 hr post) | 23.1 ± 4.8 | 28.2 ± 6.6 | 19.4 ± 3.1* |
| Retracting (pre) | 34.1 ± 3.0 | 27.3 ± 1.6 | 33.8 ± 2.1 |
| Retracting (2 hr post) | 34.4 ± 2.3 | 36.2 ± 2.9* | 41.6 ± 0.5* |
| Rate (µm/30 s) | | | |
| Extension (pre) | 1.26 ± 0.06 | 1.04 ± 0.04 | 1.34 ± 0.07 |
| Extension (2 hr post) | 1.39 ± 0.13 | 1.26 ± 0.11 | 1.45 ± 0.14 |
| Retraction (pre) | 1.30 ± 0.04 | 1.02 ± 0.07 | 1.35 ± 0.05 |
| Retraction (2 hr post) | 1.49 ± 0.15 | 1.06 ± 0.07 | 1.53 ± 0.14 |

Figure 2.6: Regulation of growth cone filopodia by MEK inhibition.

(A) Treatment of B5 neurons with the MEK inhibitor PD 98059 (40 μ M) to inhibit the protein kinase branch of PI-3K caused a significant overall increase in filopodial length as early as 5 min after addition of the drug. PD 98059 was washed out in an exchange with defined medium after 15 min of treatment. Exposure of growth cones to DMSO (0.08%), the solvent in which PD 98059 was applied, had no effect on filopodial length, and the treatment group was significantly different from the control ($p \leq 0.001$). **(B)** Treatment with PD 98059 (40 μ M) had no significant effect on filopodial number when compared to the control group ($p = 0.50$). **(C)** The time filopodia spend extending significantly increased within the first five minutes following PD 98059 (40 μ M) addition in comparison to control (7 min before drug addition) ($p \leq 0.05$). No differences were observed in the times filopodia spent stable and retracting ($p = 0.21$ and $p = 0.50$ respectively). **(D)** The filopodial extension rate was significantly increased after MEK inhibition with PD 98059 (40 μ M) in comparison to control (before drug addition, “Pre”) ($p \leq 0.05$). The filopodial retraction rate was not affected ($p = 0.98$).



filopodial length change. The number of filopodia on a growth cone was not affected by the inhibition of MEK ($p = 0.50$) (Fig. 2.6B), which was consistent with the lack of effect on filopodial number after inhibiting PI-3K. It is important to note that PD 98059 started to crystallize after 15 minutes of application, at which time the drug was washed out through an exchange with defined medium. Thus time points after the initial ten minutes have to be taken with caution, although a return of filopodial length towards pretreatment levels observed after washout of the drug indicated some reversibility of the drug treatment. Note that the tendency to crystallize precluded studies on neurite outgrowth employing this drug.

The investigation of dynamic changes in filopodial length revealed that PD 98059 significantly increased the time that filopodia spent extending from 37.4 ± 1.6 % before treatment to 44.9 ± 2.2 % after treatment ($p \leq 0.05$) (Fig. 2.6C). The time filopodia were stationary or spent retracting was not affected ($p = 0.21$ and $p = 0.50$ respectively) (Fig. 2.6C). The filopodial extension rate was significantly increased from 1.39 ± 0.12 $\mu\text{m}/30$ sec before treatment (Pre) to 1.87 ± 0.12 $\mu\text{m}/30$ sec after MEK inhibition ($p \leq 0.05$), whereas the filopodial retraction rate was not affected ($p = 0.98$) (Fig. 2.6D). These results suggested that MEK activity has a strong effect on filopodial length and might serve as a regulator of actin dynamics in *Helisoma* growth cones.

Both activities of PI-3K act together to regulate filopodial dynamics

Because the inhibition of individual targets of the lipase and kinase activities of PI-3K (Akt, ROCK, and MEK) each induced a smaller overall effect on filopodial elongation (~ 20 - 28%) than seen after PI-3K inhibition itself ($\sim 46\%$), we next wanted to investigate if MEK, Akt, and ROCK might interact to affect filopodial dynamics. To test this possibility, we treated B5

neurons with various combinations of the following inhibitors: MEK inhibitor PD 98059 (40 μ M), Akt inhibitor (80 μ M), and ROCK inhibitor Y27632 (10 μ M). Interestingly, filopodia elongated by $42.3 \pm 6.0\%$ after 15 min of combined treatment with ROCK and MEK inhibitors, and by $46.6 \pm 5.2\%$ when all three inhibitors were combined. Whereas these values were not significantly different from each other and from treatment with the PI-3K inhibitor itself, they were significantly larger than those obtained when each inhibitor was applied separately (Fig. 2.7). Note that there was no additive effect when the two kinases in the lipid kinase branch (Akt and ROCK) were inhibited simultaneously (Fig. 2.7).

Growth cone behavior is not mediated by altering $[Ca^{2+}]_i$

PI-3K and its downstream targets have been shown to regulate the intracellular calcium concentration ($[Ca^{2+}]_i$) in different systems (Blair et al., 1999; Macrez et al., 2001; Singleton and Bourguignon, 2002). An elevation in $[Ca^{2+}]_i$ in B5 growth cones results in filopodial elongation (Rehder and Kater, 1992; Cheng et al., 2002) and a slow down in neurite outgrowth (Cohan et al., 1987; Mattson and Kater, 1987). Thus it was important to investigate whether the filopodial elongation observed after inhibition of the PI-3K signaling pathway was actually due to an increase in $[Ca^{2+}]_i$ resulting from the pharmacological treatment or if the effect was Ca^{2+} -independent. Employing the calcium indicator dye, fura-2, we detected no increase in the $[Ca^{2+}]_i$ in B5 growth cones after inhibition of PI-3K with wortmannin (2 μ M), of Akt with Akt inhibitor (80 μ M) and of ROCK with Y27632 (10 μ M) (Fig. 2.8). However, because the crystals that formed 15 minutes after treatment with MEK inhibitor PD 98059 (40 μ M) were autofluorescent, we were unable to measure the $[Ca^{2+}]_i$ for this condition during the second half of the experiment. In the first 10 minutes before crystal formation, however, no increase in $[Ca^{2+}]_i$ was

detected. Depolarization of B5 neurons with a high K^+ containing medium (34 mM KCl) resulted in a significant increase in $[Ca^{2+}]_i$ in B5 growth cones from 59 ± 2 nM at rest to maximal levels of 128 ± 5 nM five minutes after treatment ($p \leq 0.001$) (Fig. 2.8), demonstrating that the calcium indicator fura-2 calcium was functional, and that a high K^+ medium elevated $[Ca^{2+}]_i$ to levels consistent with observations in previous studies on the same cell type (Rehder and Cheng, 1998).

PI-3K regulates actin dynamics

Filopodia are composed of bundles of filamentous (f-) actin (Gordon-Weeks, 1987) and the barbed ends, where net addition of monomeric actin occurs, are located towards the filopodial tip. Theoretically, filopodial elongation could result from: (i) an increase in actin polymerization at the barbed end, (ii) a decrease in retrograde flow of f-actin without affecting the normal net addition of monomeric actin at the barbed end, or (iii) a combination of both (Fig. 2.9A). In initial experiments we wanted to obtain a qualitative assessment of the contribution of these mechanisms to filopodial length changes in untreated growth cones. Blebbistatin (Bleb) was employed to inhibit the ATPase activity of nonmuscle myosin II (Straight et al., 2003) and thereby block its potential contribution to the retrograde f-actin flow (Fig. 2.9A). Addition of blebbistatin (100 μ M) resulted in a gradual increase in filopodial length over time which reached $55.5 \pm 17.9\%$ after 30 min of treatment when compared to its control (DMSO) ($p \leq 0.01$) (Fig. 2.9B), consistent with the notion that barbed end addition would continue in the absence of retrograde flow, creating a net increase in f-actin filament length (Fig. 2.9A). Cytochalasin B (Cyto B) was used to interfere with the addition of monomeric actin to the barbed end of f-actin. While Cyto B has been shown to result in the depolymerization of f-actin (Forscher and Smith, 1988), we used Cyto B at a concentration (30 nM) that had no net effect on overall

Figure 2.7: Both activities of PI-3K interact to regulate filopodial dynamics.

B5 neurons were treated with the following inhibitors either separately or in combinations, and filopodial elongation was assessed 15 min after treatment. Treatment with PI-3K inhibitor LY 294002 (25 μ M), Akt inhibitor (80 μ M), ROCK inhibitor Y27632 (10 μ M), and MEK inhibitor PD 98059 (40 μ M) induced overall filopodial elongation of $34.8 \pm 7.9\%$, $25.7 \pm 3.5\%$, $23.6 \pm 2.3\%$, and $18.7 \pm 3.4\%$, respectively. Filopodia elongated by $42.3 \pm 6.0\%$ when one kinase each of the lipid- and protein kinase branch was inhibited together (ROCK and MEK inhibitors). Simultaneous inhibition of Akt, ROCK and MEK resulted in filopodial elongation of $46.6 \pm 5.2\%$ and had no significant additional effect over inhibiting ROCK and MEK simultaneously. The combinatorial effects were not significantly different from treatment with the PI-3K inhibitor itself ($p = 0.08$), but significantly larger than when Akt inhibitor (80 μ M) ($p \leq 0.01$), ROCK inhibitor, Y27632 (10 μ M) ($p \leq 0.001$), and MEK inhibitor, PD 98059 (40 μ M) ($p \leq 0.001$) were applied separately. Note that there was no additive effect when the two kinases in the lipid kinase branch (Akt and ROCK) were inhibited simultaneously. The control condition denotes a final concentration of 0.125% DMSO (as used for LY 294002 (25 μ M)), and this control was not significantly different from controls for the other solvents used (not shown).

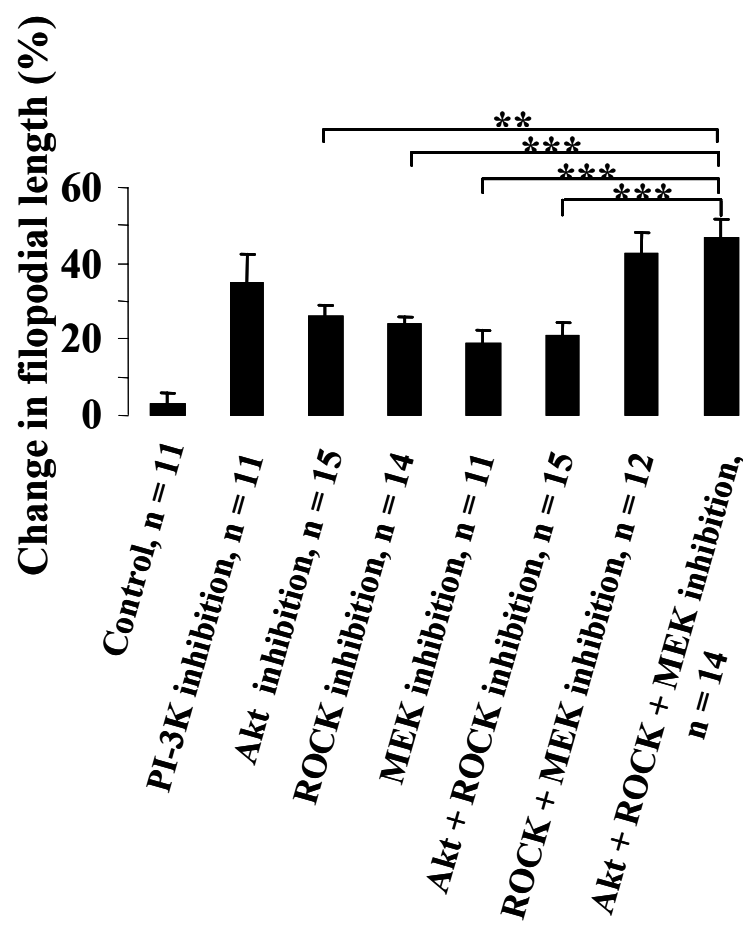


Figure 2.8: Growth cone behavior was not mediated by altering $[Ca^{2+}]_i$.

Measurements of $[Ca^{2+}]_i$ with the Ca^{2+} indicator fura-2 in response to various treatments used in this study show that the inhibition of PI-3K and its downstream targets had no significant effect on $[Ca^{2+}]_i$. A control treatment with high KCl (34 mM) increased $[Ca^{2+}]_i$ to maximum levels of 128 nM after 5 min ($p \leq 0.001$).

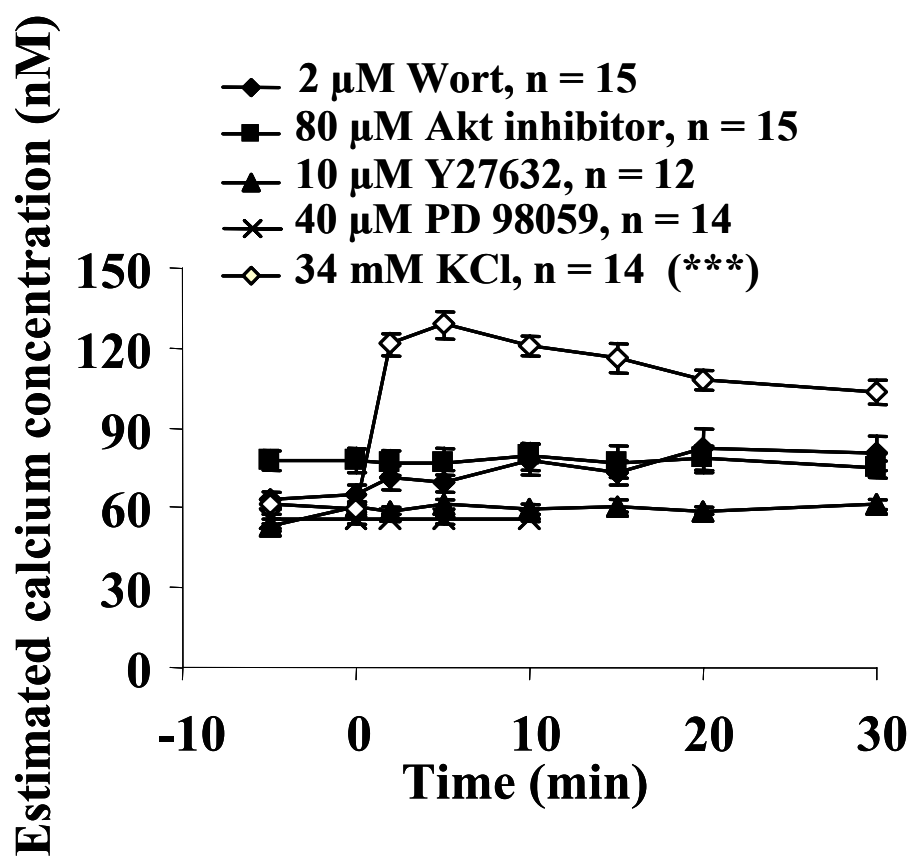
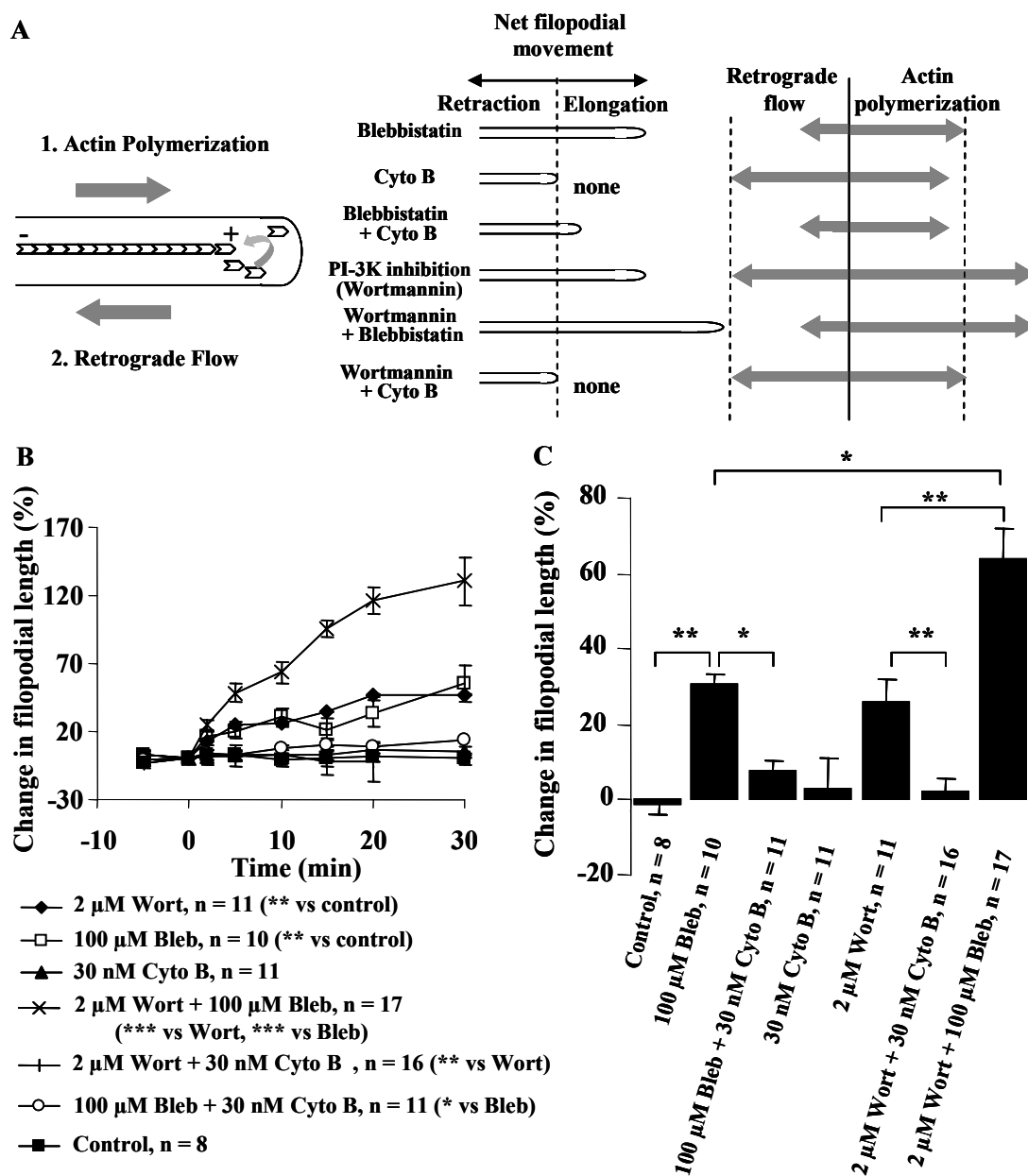


Figure 2.9: Filopodial elongation resulting from PI-3K inhibition requires actin polymerization.

(A) Model of filopodial length changes. Filopodial length is mainly regulated by actin polymerization/depolymerization events at the barbed end and the retrograde flow by which f-actin is transported centrally (left). The curved arrow indicates actin polymerization onto the barbed (+) end of f-actin, which would result in filopodial elongation. Arrows pointing to the right symbolize actin polymerization and arrows pointing to the left symbolize retrograde flow. The middle panel shows the net effect of various experimental treatments on overall filopodial length. The right panel shows the hypothesized relative contributions (indicated by the length of the arrows) of actin polymerization and retrograde flow on filopodial length under these experimental conditions. See text and (B) for details. **(B)** Inhibition of ATPase activity of nonmuscle myosin II with blebbistatin (100 μ M) resulted in a gradual increase in filopodial length over time that was significant when compared to its control (DMSO) ($p \leq 0.01$). The inhibitor of actin polymerization, cytochalasin B, at the low concentration of 30 nM had no net effect on filopodial length ($p = 0.67$). Combined treatment with cytochalasin B (30 nM) and blebbistatin (100 μ M) blocked filopodial elongation induced by blebbistatin treatment alone ($p \leq 0.05$). Inhibition of PI-3K with wortmannin (2 μ M) resulted in net filopodial elongation, and this effect was of a similar magnitude as observed with blebbistatin treatment. Wortmannin (2 μ M) in the presence of blebbistatin (100 μ M) induced a larger effect on filopodial elongation when compared to wortmannin and blebbistatin applied separately ($p \leq 0.001$). Filopodial elongation observed after wortmannin treatment was blocked by 10 minutes preincubation with 30 nM cytochalasin B ($p \leq 0.01$). **(C)** Effect on filopodial length from (B) after 10 minutes of treatment.



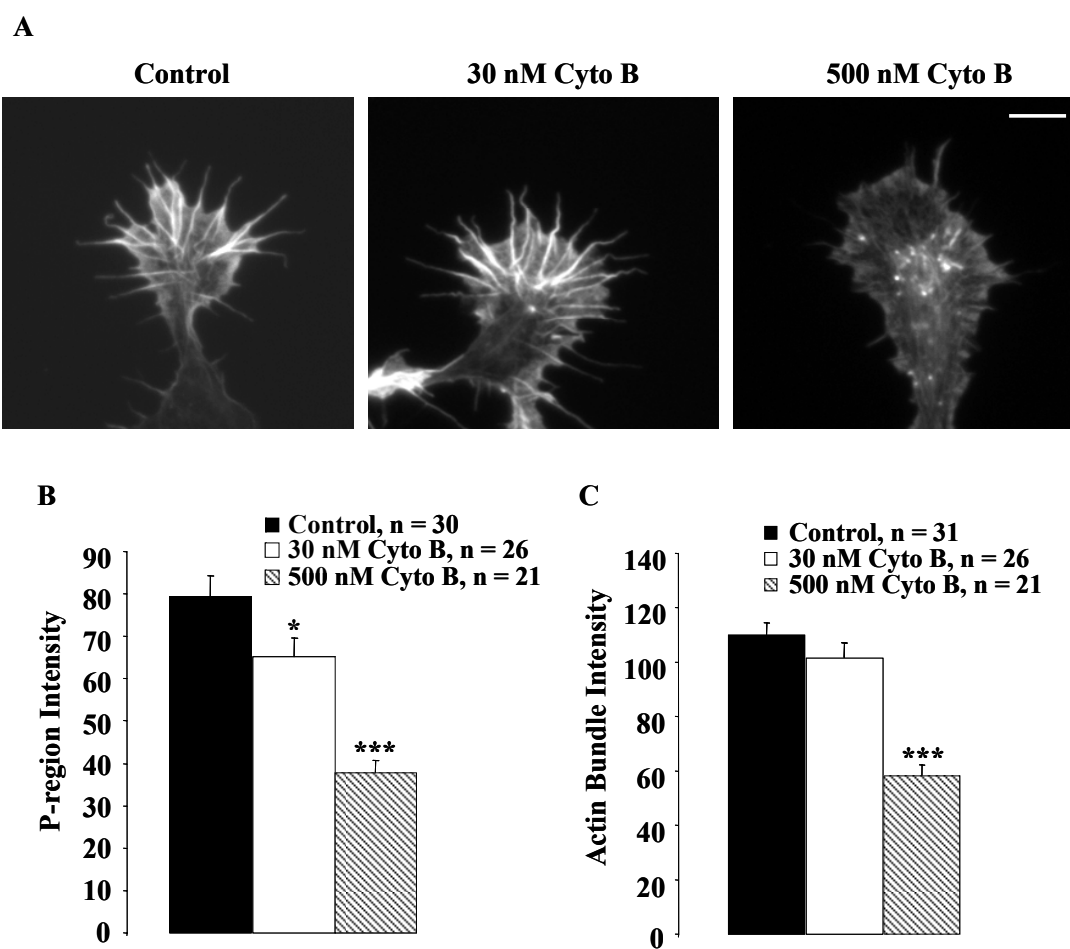
filopodial length (Fig. 2.9 and Geddis et al., 2004). This result suggested that barbed end addition was slowed by Cyto B to a degree that it was equal and opposite to the effect of retrograde flow. When neurons were incubated in blebbistatin plus Cyto B the change in filopodial net length seen with blebbistatin treatment alone was significantly blocked ($p \leq 0.05$) (Fig. 2.9B and C), suggesting that when plus-end addition cannot occur, the filopodial elongation that is seen with blebbistatin alone is blocked, demonstrating the important role of actin polymerization for filopodial elongation.

To confirm that the total f-actin content was not affected by the treatment with 30 nM Cyto B, we employed a fluorescently labeled mushroom toxin, rhodamine phalloidin, which binds to f-actin. Overall, growth cones treated with 30 nM Cyto B for 30 minutes appeared no different from control growth cones (Fig. 2.10A). However, treatment with a higher concentration of Cyto B (500 nM) resulted in an obvious decrease in f-actin staining and a loss of filopodia (Fig. 2.10A). Quantification of the intensity of f-actin staining within the lamellipodial P-region of growth cones, however, revealed a small decrease in the intensity of staining in growth cones treated with 30 nM Cyto B when compared to control growth cones (from 79.4 ± 4.8 to 65.1 ± 4.4 fluorescence units; Fig. 2.10B; $p \leq 0.05$, t-test). Treatment with 500 nM Cyto B showed a large reduction in f-actin staining in the P-region (to 37.7 ± 3.0) (Fig. 2.10A and B; $p \leq 0.001$, t-test). When f-actin content of individual f-actin bundles (forming the core of filopodia) was measured (see Methods for details), no difference between control growth cones and those treated with 30 nM Cyto B was found (Fig. 2.10C; $p = 0.23$, t-test). Growth cones treated with 500 nM Cyto B, however, showed a significant loss in actin bundle intensity (from 110.0 ± 4.4 to 58.2 ± 3.9 ; Fig. 2.10C; $p \leq 0.001$, t-test). These results suggested that although 30 nM Cyto B slightly reduced the amount of f-actin within the P-region of the growth cone, the f-actin content within filopodia was not measurably affected.

We next wanted to use the tools developed above to investigate whether PI-3K inhibition

Figure 2.10: A low concentration of cytochalasin B (30 nM) does not affect filopodial actin bundles, but slightly reduces overall f-actin staining intensity within the p-region.

Growth cones received one of three treatments (L-15 medium exchange (Control), 30 nM Cyto B in L-15, or 500 nM Cyto B in L-15) for 30 minutes and were subsequently fixed in paraformaldehyde/glutaraldehyde and reacted with rhodamine phalloidin. **(A)** Growth cones that received the 30 nM Cyto B treatment look indistinguishable from control growth cones regarding overall morphology, as well as number and intensity of fluorescent F-actin bundles visible in the growth cone. Note the noticeable loss of f-actin staining and filopodia in growth cones treated with 500 nM Cyto B. Scale: 10 μ m. **(B)** Intensity of f-actin staining in the p-region was significantly reduced (from 79.4 ± 4.8 to 65.1 ± 4.4) by treatment with 30 nM Cyto B ($p \leq 0.05$ as compared to Control). Treatment with 500 nM Cyto B reduced the intensity of f-actin staining within the p-region to an even greater extent (to 37.7 ± 3.0 ; $p \leq 0.001$ as compared to Control). **(C)** Actin bundle intensity was not affected by treatment with 30 nM Cyto B ($p = 0.23$ as compared to Control). However, treatment with 500 nM Cyto B significantly reduced actin bundle intensity from 110.0 ± 4.4 to 58.2 ± 3.9 ($p \leq 0.001$ as compared to Control).



resulted in filopodial elongation by increasing g-actin addition to the barbed end and/or by inhibiting f-actin's retrograde flow. Because the magnitude of filopodial elongation seen after treatment with either wortmannin (to block PI-3K) or blebbistatin (to block retrograde flow) was similar, (approximately 25-30% for both at 10 min) (Fig. 2.9B and C) it was possible that PI-3K inhibition blocked retrograde flow. To test this hypothesis, wortmannin was applied in the presence of blebbistatin. Interestingly, the effect on filopodial elongation was as large or even larger than the numerical sum of the effects seen with either wortmannin or blebbistatin treatment alone, reaching a $63 \pm 8\%$ increase in filopodial length after 10 min ($p \leq 0.01$ and $p \leq 0.05$ compared to wortmannin and blebbistatin alone) (Fig. 2.9B and C). This observation was consistent with a mechanism by which wortmannin, increased filopodial length by increasing actin polymerization at the barbed end. To investigate this likely mechanism further, we inhibited PI-3K in the presence of Cyto B with the assumption that if PI-3K inhibition increased actin polymerization onto the barbed end, we should be able to block the effect on filopodial elongation in the presence of Cyto B. Whereas wortmannin caused an increase in filopodial length of $25.8 \pm 6.4\%$ after 10 min, this effect was inhibited significantly after a 10 minutes preincubation with 30 nM Cyto B ($p \leq 0.01$) (Fig. 2.9B and C). This result suggested that actin polymerization is the major factor by which PI-3K inhibition causes filopodial elongation. Moreover the observation that there was no significant difference in the effects of treatment with Cyto B plus wortmannin and with Cyto B by itself further supported the hypothesis that the PI-3K inhibitor, wortmannin, increased filopodial length by increasing actin polymerization at the barbed end.

Discussion

Phosphatidylinositol-3-kinase (PI-3K) is central to many signaling pathways and has been shown to function in cellular events such as cell survival, mitogenesis, differentiation and motility (Duronio et al., 1998; Krasilnikov, 2000; Cantrell, 2001; Rodgers and Theibert, 2002). PI-3K can be activated by receptor tyrosine kinases, non-receptor tyrosine kinases, and G-protein coupled receptors (Stephens et al., 1994; Stoyanov et al., 1995; Lopez-Illasaca et al., 1997; Duronio et al., 1998; Vanhaesebroeck and Waterfield, 1999; Cantrell, 2001) but the mechanism by which PI-3K affects neurite outgrowth is not well understood. Here we set out to investigate whether the effect of PI-3K on neurite outgrowth might be explained by its action on the actin cytoskeleton in neuronal growth cones.

PI-3K signaling events regulate filopodial dynamics and neurite outgrowth

Inhibition of PI-3K with wortmannin induced a rapid increase in filopodial length. Filopodial elongation was observed at micromolar concentrations of wortmannin at which this drug is not solely specific for PI-3K but can also affect PI-4K (Rodgers and Theibert, 2002). We do not think that this activity interfered with PI-3K inhibition, because the same increase in filopodial length was observed after treatment with a second, more specific PI-3K inhibitor, LY 294002. The fact that two different specific inhibitors induced the same effect on filopodial length strongly suggests that PI-3K activity regulates filopodial dynamics. The study of the dynamic parameters of filopodia, by tracking individual filopodia at 30 second intervals, showed that the overall filopodial elongation induced by PI-3K inhibition was produced by an increase in the time individual filopodia spent extending and by an increase in the extension rate of individual filopodia.

In addition to its effect on filopodia, treatment with the specific PI-3K inhibitor, LY 294002, also caused a significant decrease in the rate of neurite outgrowth in the first and second hour of PI-3K inhibition (Fig. 2.2). This decrease in outgrowth likely resulted from a mechanism other than affecting filopodial motility, since filopodial dynamic parameters were virtually unchanged before and two hours after treatment with the PI-3K inhibitor. Instead, it is reasonable to assume that the PI-3K inhibitor acted on microtubules, because neuronal growth rates are dependent on microtubule dynamics (Bamburg et al., 1986; Gordon-Weeks, 1991) and recent evidence suggests that PI-3K activity can regulate microtubules (Zhou et al., 2004; Zhou and Snider, 2005).

Our results on the role of PI-3K signaling on neurite outgrowth are in agreement with studies on other cell types. Pharmacological inhibition of PI-3K reduces neurite outgrowth in mouse DRG and hippocampal neurons (Wu et al., 1998; Edstrom and Ekstrom, 2003), as well as in PC12 cells (Jackson et al., 1996). On the other hand, PI-3K activation by NGF has been shown to increase axon outgrowth in DRG neurons by inactivation of GSK-3 β and the involvement of the microtubule plus end binding protein APC (Zhou et al., 2004), although NGF stimulation has also been reported to signal via the MAPK pathway (Goold and Gordon-Weeks, 2005). Taken together, inhibition of PI-3K elicits filopodial elongation and a concomitant decrease in neurite advance, a phenomenon we have previously described to occur in response to treatment with nitric oxide (Trimm and Rehder, 2004) and termed ‘slow down and search’ behavior.

ROCK, Akt, and MEK regulate filopodial dynamics and neurite outgrowth

PI-3K can be activated by receptor- and non-receptor tyrosine kinases, as well as by G-protein coupled receptors (Stephens et al., 1994; Stoyanov et al., 1995; Lopez-Illasaca et al.,

1997; Duronio et al., 1998; Vanhaesebroeck and Waterfield, 1999; Cantrell, 2001). PI-3K, in turn, has two modes of action. It functions as a lipid kinase by phosphorylating the 3'-OH position on the inositol ring of several phosphoinositides, thereby producing inositol lipid products, which are important mediators of intracellular signaling (Duronio et al., 1998; Vanhaesebroeck and Waterfield, 1999; Cantrell, 2001). This signaling pathway leads to the stimulation of guanine nucleotide exchange factors for Rho-GTPases, as well as to the activation of the PH-domain containing kinase, PDK-1, which in turn activates Akt (Alessi et al., 1997; Downward, 1998; Meier and Hemmings, 1999; Vanhaesebroeck and Alessi, 2000) (Fig. 2.3). Secondly, PI-3K functions as a protein kinase resulting in the activation of the Ras/Raf/Erk pathway (Bondeva et al., 1998; Vanhaesebroeck and Waterfield, 1999; Krasilnikov, 2000).

Given the complexity of PI-3K signaling, we here concentrated on three key kinases downstream of PI-3K activity, namely ROCK, Akt, and MEK. Inhibition of any of these kinases led to an overall increase in filopodial length, which resulted from an increase in both the extension rate and the time filopodia spent extending. This finding was somewhat unexpected, since it suggested that all three kinases were able to affect filopodial length and could play a role in regulating growth cone dynamics during neuronal pathfinding (Fig. 2.3). The finding that the simultaneous inhibition of Akt, ROCK and MEK mimicked the inhibition of PI-3K itself, and induced a stronger filopodial elongation than when each kinase was inhibited individually, suggested that the three pathways might interact to regulate actin dynamics. Interestingly, simultaneous inhibition of the two kinases in the lipid kinase branch of PI-3K activity, namely ROCK and Akt, had no additive effect, indicating that their actions may have a common downstream element. Inhibition of one member each of the lipid kinase (ROCK) and protein kinase pathways (MEK), however, resulted in an additive effect, suggesting that ROCK and

MEK might affect filopodial length by different pathways that converge on elements regulating actin dynamics (Fig. 2.3).

Akt is crucial to cell survival and the inhibition of Akt has been demonstrated to cause cell death (Dudek et al., 1997). Since cell death is a process occurring on a much longer time scale than the effects on filopodial length, we were less concerned that the filopodial response could be a consequence of apoptosis in response to Akt inhibition. Rather, the time scale of minutes indicated a direct signaling pathway involving the cytoskeleton locally.

In addition to the effects on filopodial behavior, we found that inhibition of two of the downstream targets of the lipid kinase pathway, Akt or ROCK, resulted in a decrease in neurite outgrowth in the second hour following drug addition (Fig. 2.5A and B). This late onset of an effect on neurite outgrowth, compared to an effect on filopodial elongation observed within minutes, suggested that filopodial elongation could occur without a concomitant decrease in neurite outgrowth. As such, the slow-down and search behavior seen in this study can be divided into two behaviors: “slow-down” and “search” (Fig. 2.11). As observed with both ROCK and Akt inhibition, it is possible for a growth cone to elongate its filopodia over a short time course (the “search” behavior) while maintaining a constant outgrowth speed. Yet, at other times these behaviors can be coupled into the “slow-down and search” behavior that is seen with PI-3K inhibition (Fig. 2.1B, Fig. 2.2) in the first hour of drug addition. Given that the treatments in this study were long-term and may not reflect shorter and probably more physiologically relevant episodes of kinase activation/inhibition, one could envision scenarios in which changes in filopodial length and neurite outgrowth rates could either be regulated independently or in conjunction, depending on the signaling cues received by the pathfinding growth cone. RhoA, an activator of ROCK, has been shown to have opposite effects on neurite outgrowth depending

on the cell type and the stage of outgrowth studied (Kranenburg et al., 1997; Threadgill et al., 1997; Kuhn et al., 1999; Sebok et al., 1999). In agreement with ROCK promoting neurite outgrowth, inactivation of RhoA has been shown to slow the rate of neurite outgrowth in NGF-primed differentiating PC12 cells (Sebok et al., 1999).

While we found that inhibition of Akt resulted in a slowing of neurite outgrowth, other recent studies reported the opposite effect, namely that expression of a dominant inhibitory form of Akt increased NGF induced neurite outgrowth in PC12 cells (Bang et al., 2001; Piiper et al., 2002). This difference in results may be explained by the fact that while we investigated neurite outgrowth on already extended neurites, the other studies focused on neurite initiation. It is, thus, possible that Akt may affect neurite initiation and neurite maintenance differently, as has been reported for RhoA (Sebok et al., 1999).

PI-3K signaling events alter filopodial length by regulating actin dynamics

Changes in filopodial length mainly result from a change in the balance of actin polymerization and depolymerization at the ends of bundled f-actin polymers in filopodia, and by altering the rate of retrograde f-actin flow (Lin et al., 1996; Mallavarapu and Mitchison, 1999). In an attempt to determine which of these mechanisms were involved in producing filopodial elongation after PI-3K inhibition, we used several drugs known to interfere with actin polymerization (cytochalasin B) and myosin II activity (blebbistatin), the latter being implicated in driving the retrograde actin flow (Brown and Bridgman, 2003). Our finding that the filopodial elongation induced by PI-3K inhibition was blocked after pretreatment with cytochalasin B demonstrated that PI-3K activity controls actin dynamics. More specifically, the results are in agreement with the hypothesis that inhibition of PI-3K increases actin polymerization at the

barbed end, although possible effects of PI-3K on actin depolymerization at the pointed end cannot be ruled out. Alternatively, elongation could come about by a decrease or stoppage of retrograde flow (Jay, 2000) and no change at the barbed end. This possibility was made less likely by the finding that inhibition of the myosin II ATPase activity with blebbistatin, which would block retrograde flow and result in an increase in filopodial length, showed an additive affect when combined with PI-3K inhibition, resulting in an increase in filopodial length of greater than 60%. Taken together our results suggest that while filopodial elongation can be achieved independently by inhibiting myosin II activity or by increasing the addition of g-actin to the barbed end, the filopodial elongation in response to PI-3K inhibition can be explained best by an increase in actin polymerization. This result is supported further by a report on retinal ganglion neurons in which filopodial elongation elicited by inhibition of either ROCK or myosin II were additive, making it unlikely that ROCK acted through myosin II (Gehler et al., 2004).

Actin associated proteins, such as profilin and cofilin, control actin dynamics (Pollard and Cooper, 1986; Stossel, 1989). Profilin inhibits actin assembly at the barbed end by binding and sequestering actin monomers (Stossel, 1989). Phosphoinositides prevent the profilin-actin monomer association by binding to profilin, with Ptdins(3,4)P₂ (PIP₂) having a higher affinity than Ptdins(3,4,5)P₃ (PIP₃) (Goldschmidt-Clermont et al., 1991; Lu et al., 1996). Moreover, profilin affinity for actin monomers has been shown to be increased by PI-3K dependent phosphorylation (Sathish et al., 2004). Therefore filopodial elongation induced by PI-3K inhibition could be due to an increase in profilin-PIP₂ binding and to a decrease in phosphorylated profilin. Actin polymerization could also be increased by preventing barbed end capping through profilin binding proteins of the Ena/VASP family (Bear et al., 2002; Krause et al., 2003) which are localized to the tips of filopodia (Lanier et al., 1999).

Filopodial elongation observed after ROCK inhibition is consistent with two recent studies on chick retinal neurons, in which BDNF binding to p75 neurotrophin receptors induces filopodial elongation by inactivating the ROCK effector, RhoA in retinal ganglion neurons (Gehler et al., 2004; Gehler et al., 2004). Filopodial elongation also resulted when ROCK was inhibited directly. Moreover, the effect of BDNF was shown to be mediated through an activation of the complex cofilin - actin-depolymerizing factor (referred to as ADF/cofilin) and not through an action of ROCK on myosin II activity. ADF/cofilin is an important regulator of actin dynamics (Gungabissoon and Bamburg, 2003), as it disassembles actin monomers from the pointed end and severs actin filaments, thereby producing more barbed ends in the process (Chen et al., 2000). Whether activation of ADF/cofilin is involved in filopodial elongation in response to PI-3K inhibition in B5 neurons is presently unclear.

A calcium-independent form of slow down and search behavior

In an earlier study that investigated the effect of nitric oxide (NO) on growth cone behavior, we described the increase in filopodial length coupled with a slow-down in neurite advance as ‘slow-down and search’ behavior (Trimm and Rehder, 2004). Interestingly, NO acted via soluble guanylyl cyclase and led to an increase in the free intracellular calcium concentration $[Ca^{2+}]_i$. In the present study, we performed Ca^{2+} -imaging experiments and found no measurable changes in $[Ca^{2+}]_i$ with any of the drug treatments, suggesting that a ‘slow-down and search’ behavior can also be elicited in a Ca^{2+} -independent fashion. Thus, a Ca^{2+} dependent (NO) and a Ca^{2+} independent (PI-3K) pathway appear to converge onto cytoskeletal regulators to similarly affect filopodial dynamics and neurite outgrowth. Actin binding proteins such as ADF/cofilin and annexin might serve as such convergence points, as they have been shown to be regulated by

Ca^{2+} , phosphatidylinositol binding and phosphorylation/dephosphorylation events (Bamburg, 1999; Hayes et al., 2004).

Filopodial length and number can be regulated independently

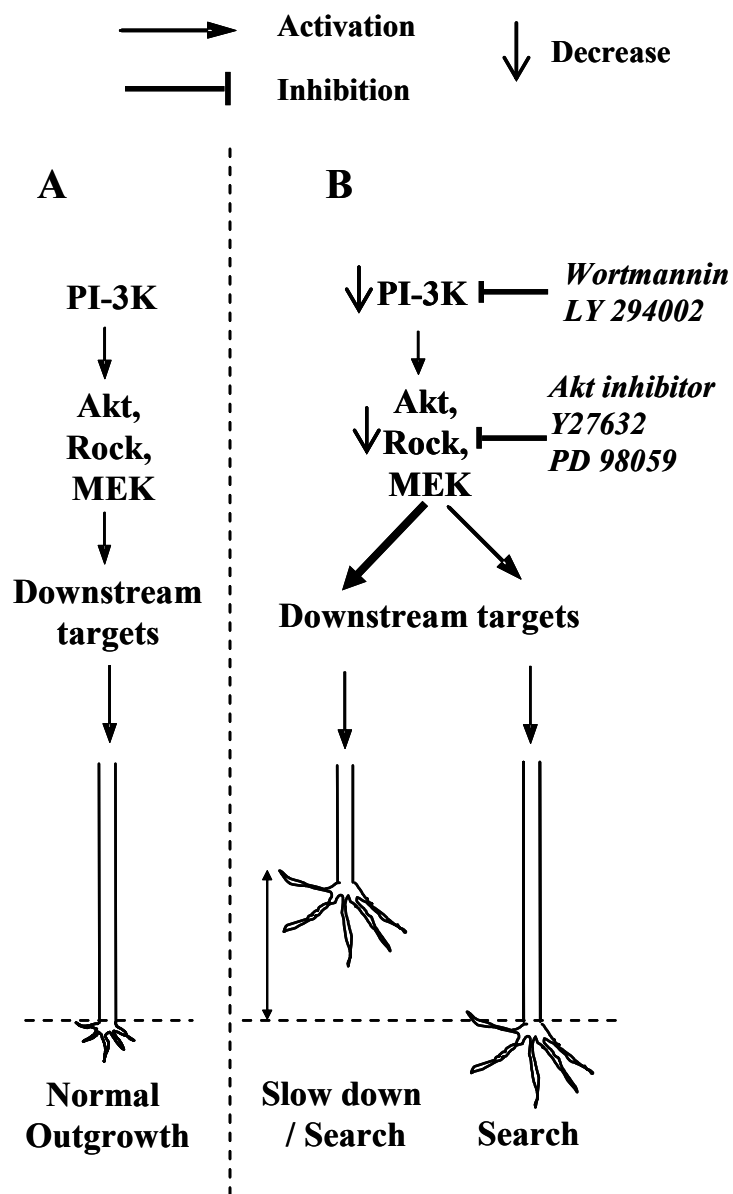
Interestingly, inhibition of PI-3K and kinases downstream had no significant effects on another morphological growth cone parameter, filopodial number. In previous studies, we had described that filopodial elongation was often coupled to a decrease in filopodial number (Van Wagenen and Rehder, 1999; Cheng et al., 2000). The present study provides strong evidence that the parameters length and number can be regulated independently from each other. This may be of significance to a navigating growth cone, which would be able to increase its action radius by elongating its filopodia without losing spatial resolution through a decrease in the number of sensors. We observed a similar uncoupling in filopodia length and number after local uncaging of Ca^{2+} in growth cones (Cheng et al., 2002), which resulted in a transient elongation of filopodia for approximate 30 min. in response to an increase in intracellular Ca^{2+} lasting several seconds.

CONCLUSIONS

Taken together, our results demonstrate that signaling events leading to the inhibition of PI-3K resulted in an increase in filopodial length and a decrease in neurite outgrowth, and as such could act as a signal for a slow-down and search behavior (Fig. 2.11). Inhibition of key kinases located downstream of PI-3K activity also resulted in filopodial elongation (ROCK, Akt, and MEK) and in a decrease in neurite advance (ROCK and Akt), consistent with the notion that PI-3K might act through these kinases to affect growth cone motility. In extension, other signaling pathways

Figure 2.11: Model of regulation of neurite outgrowth and filopodial motility by PI-3K.

(A) Baseline PI-3K activity leads to downstream signaling events that result in growth cones advancing with ‘baseline’ speed and having filopodia of certain lengths. **(B)** When PI-3K or key kinases in the proposed pathway downstream of PI-3K are inhibited or otherwise reduced in their activity, growth cones undergo a “slow-down and search” behavior, characterized by filopodial elongation and a concomitant decrease in the rate of neurite advance. Note that depending upon the duration or intensity with which a pathway is inhibited, the growth cone may respond with either the combined slow down and search behavior or with filopodial elongation (“search”) alone.



converging on these kinases are expected to have predictable effects on neurite outgrowth and filopodial behavior. Filopodial elongation in response to PI-3K inhibition was shown to result largely from an increase in actin polymerization, and not from an inhibition of the retrograde flow. The detailed pathways that link the activity of these kinases to changes in the growth cone cytoskeleton will have to be investigated in future studies.

Chapter 3 : Nitric Oxide Release from a Single Cell Affects Filopodial Motility on Growth Cones of Neighboring Neurons

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Abstract

Nitric oxide (NO), a gaseous messenger, has been reported to be involved in a variety of functions in the nervous system, ranging from neuronal pathfinding to learning and memory. We have shown previously that the application of NO via NO donors to growth cones of identified *Helisoma* buccal neurons B5 *in vitro* induces an increase in filopodial length, a decrease in filopodial number, and a slowing in neurite advance. It is unclear, however, whether NO released from a physiological source would affect growth cone dynamics. Here we used cell bodies of identified neurons known to express the NO synthesizing enzyme nitric oxide synthase (NOS) as a source of constitutive NO production and tested their effect on growth cones of other cells in a sender-receiver paradigm. We showed that B5 cell bodies induced a rapid increase in filopodial length in NO-responsive growth cones, and that this effect was blocked by the NOS inhibitor 7-NI, suggesting that the effect was mediated by NO. Inhibition of soluble guanylyl cyclase (sGC) with ODQ blocked filopodial elongation induced by B5 somata, confirming that NO acted via sGC. We also demonstrate that the effect of NO was reversible and that a cell releasing NO can affect growth cones over a distance of at least 100 μm . Our results suggest that NO released from a physiological source can affect the motility of nearby growth cones and thus should be considered a signaling molecule with the potential to affect the outcome of neuronal pathfinding *in vivo*.

Introduction

Nitric oxide (NO), a gaseous messenger, has been described to play an important role during neuronal development, such as in the formation of retinal projections *in vivo* (Williams et al., 1994; Cramer et al., 1996; Gibbs and Truman, 1998; Cramer and Sur, 1999; Ernst et al.,

1999; Wildemann and Bicker, 1999; Wu et al., 2000), but also in neuronal differentiation and neurite outgrowth in general (Hess et al., 1993; Peunova and Enikolopov, 1995; Hindley et al., 1997; Poluha et al., 1997; Nakaya et al., 2000; Rialas et al., 2000; Gallo et al., 2002). During the development of the grasshopper embryo, NO, potentially released from epithelial cells facing the basal lamina of the developing antenna, has been shown to regulate axonal pathfinding of antennal pioneer neurons (Seidel and Bicker, 2000; Bicker, 2007). We have shown previously that NO donors applied to identified *Helisoma* B5 neurons grown *in vitro* induce an increase in filopodial length and a concomitant decrease in neurite outgrowth (Van Wagenen and Rehder, 1999; Trimm and Rehder, 2004; Welshhans and Rehder, 2005), suggesting that some of the developmental effects of NO described in the literature might result from NO acting on neuronal growth cones. A follow up study, in which NO donors were applied focally to B5 growth cones, indeed confirmed that NO regulates filopodial motility locally at the growth cone and that the NO signaling pathway included soluble guanylyl cyclase (sGC), protein kinase G (PKG), cyclic adenosine diphosphate ribose (cADPR) and intracellular calcium release from ryanodine sensitive stores (Welshhans and Rehder, 2005; Welshhans and Rehder, in press). While the application of NO donors has proven to be a practical approach to test effects of NO in numerous preparations, it is not known whether NO released physiologically from other cells would affect growth cone motility as well. It has been shown both *in vivo* and *in vitro* that cellular release of NO can have measurable effects on neighboring cells, such as acting as a modulator of neuronal excitability (Elphick et al., 1995; Park et al., 1998).

The ultimate determination whether **cellular** release of NO can affect growth cone motility in another cell by acting in a paracrine fashion has not yet been made. In this study we used cell bodies of identified neurons known to express the NO synthesizing enzyme nitric oxide synthase (NOS) as

sources of constitutive NO production and paired them with growth cones to be tested for their response to NO. To facilitate the testing, neuronal somata adhering to a poly-L-lysine coated microelectrode were maneuvered into the vicinity of growth cones (distances ranging from 20-150 μm) and filopodial dynamics were recorded before and at defined times after stimulation. We here, to our knowledge for the first time, demonstrate that neuronally released NO is effective in altering filopodial motility on growth cones of neighboring neurons. This finding not only suggests a role for NO in neuronal pathfinding, but also provides a straightforward physiological paradigm to study the effects of NO on cell motility in general.

Methods and Materials

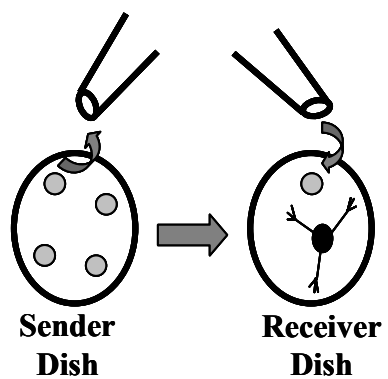
Neuronal culture and image acquisition

Identified B5 and B19 neurons were dissected from *Helisoma trivolvis* buccal ganglia and plated for neuronal culture. Neurons to be investigated for the effect of NO on growth cone morphology (referred to as “Receivers”) were plated on poly-L-lysine (hydrobromide, MW 70,000-150,000, 0.25mg/ml, Sigma, St. Louis, MO) coated glass coverslips glued to the bottoms of a 35 mm cell culture dishes (Falcon 1008). These cells were grown overnight at room temperature and used 24-48 hours after plating (Fig. 3.1A). Cells used as sources of NO (referred to as “Senders”) were obtained by placing freshly harvested cell bodies into hemolymph-coated glass dishes according to Spencer et al. (2000) to prevent them from adhering to the substrate. These cells were kept at room temperature and used within 24 hours after plating (Fig. 3.1A). “Receiver” cells were plated into conditioned medium (CM), prepared by incubating snail brains in modified Leibowitz L-15 medium (Gibco, Grand Island, NY) for 3-4 days. L-15 medium was prepared to contain final concentration of: 40 mM NaCl, 1.7 mM KCl, 1.5 mM MgCl_2 , 4.1 mM CaCl_2 , 10

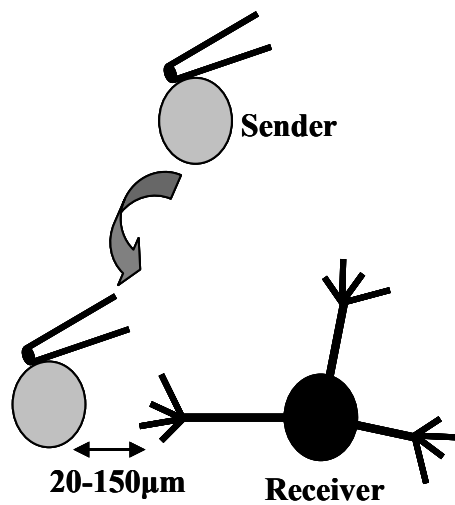
Figure 3.1: Schematic of the experimental design.

(A) Sender dishes (coated with hemolymph) contain neuronal cell bodies to be used as NO “senders”, whereas receiver dishes are coated with poly-L-lysine and contain conditioned medium to support neurite outgrowth from neurons to be tested in the sender/receiver paradigm. Senders are transferred to the receiver dish with a fire-polished micropipette. **(B)** Sender neurons are attached to a poly-L-lysine coated glass micropipette which is subsequently positioned by a motorized micromanipulator to hold the sender at variable distances, ranging from 20-150 μm from a “receiver” growth cone.

A



B



mM HEPES, 50 μ g/ml gentamycin, 0.15 mg/ml glutamate in distilled water (pH=7.3) (Cohan et al., 2003). A fire-polished micropipette, connected hydraulically via plastic tubing to a micrometer syringe (Gilmont Instruments), was used to transfer an individual “Sender” cell body into the culture dish containing the neuron to be tested. The “sender” cell body was subsequently maneuvered into the proximity (20-150 μ m) of the “receiver” growth cone by using a poly-L-lysine-coated microelectrode attached to a micromanipulator (Spencer et al., 2000) (Fig. 3.1B). Growth cones were viewed under a 100x oil immersion objective on a Sedival (aus Jena, Germany) microscope. A regular CCD camera (Model: C-72, MIT Dage, Michigan City, IN) was used to capture images, which were digitized on a frame grabber (Scion LG-3, Scanalytics, Fairfax, VA) and analyzed with “Scion Image” software (Scion Corporation; Frederick, Maryland) on a Hewlett-Packard Pavilion (Hewlett-Packard Company, Palo Alto, CA).

Pharmacological agents

7-NI (7-Nitroindazole, nNOS inhibitor) and ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one, soluble guanylyl cyclase inhibitor) were dissolved in dimethylsulfoxide (DMSO, Sigma) to obtain stock concentrations of 10 and 20 mM respectively. All drugs were purchased from Calbiochem (San Diego, CA).

Design and analysis of growth cone dynamics

Observation of filopodial length and number were performed at defined times before and after moving a “sender” cell body into the vicinity of the growth cone to be tested. Other growth cones on the same cell, but located at considerable distances from the growth cone to be tested, were used as controls (not stimulated by the sender) and labeled as “Non-receiver growth cone”. In experiments involving 20 min of preincubation with a pharmacological agent, drugs were

premixed in 2 mL of L-15 medium to obtain final concentrations, and introduced to the dish by complete medium exchange. Growth cone dynamics were analyzed during the preincubation period to determine whether any of the drugs would have an effect by itself. Images were captured before (-5 and 0 min) and at defined times (2, 5, 10, 15, 20, and 30 minutes) after maneuvering the sender into the vicinity of the growth cone.

Digitized images of growth cones were analyzed by measuring the length of all individual filopodia from their tip to the edge of the central domain, preventing potential incorrect measurement influenced by lamellipodial dynamics. Filopodial length was measured for each filopodium on a growth cone from phase contrast images using the software package “Scion Image” and is expressed as mean filopodial length. To compensate for the variability observed in growth cone size and filopodial length between different growth cones, these values were expressed as a percentage change normalized to the time point $t = 0$ just prior to any manipulation.

Statistical analysis

Analysis of variance (repeated measures ANOVA with time as repeated measures when appropriate), and t -test for independent samples with SuperANOVA (Abacus Concepts, Berkeley, CA) were performed to determine statistical significance between conditions. Fisher’s protected least significant difference (PLSD) test was used as post-hoc analysis of preplanned comparisons. Significant differences are indicated as: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

Results

Growth cone dynamics are affected by a B5 sender/receiver paradigm

To investigate if the physiological release of NO from a neuron could act as a messenger and affect nearby growth cones, we made use of the fact that B5 neurons express NOS (Van Wagenen and Rehder, 2001), produce NO (Ivanova and Rehder, unpublished results), and can therefore be used as a physiological source of NO. Other cells, such as the B19 neuron, were previously demonstrated to not contain significant NOS immunoreactivity and therefore have been used successfully as negative controls (Van Wagenen and Rehder, 2001). To make the stimulation paradigm as flexible as possible, we used a poly-L-lysine coated micropipette, which adheres well to any cell body and thus provides a handle by which a cell body can be moved easily within the dish (Spencer et al., 2000). Using this approach, cell bodies can be positioned and held at variable distances from growth cones of other cells growing in the culture dish. The “sender” is subsequently maneuvered into position close to a growth cone to be tested (referred to as the “receiver”). Stimulation of a B5 receiver growth cone by a B5 sender positioned at a distance of 20-30 μm resulted in a significant and rapid elongation of filopodia when compared to the control condition, in which a poly-L-lysine coated micropipette was positioned at the same distance alone ($p \leq 0.001$) (Fig. 3.2A and B). The elongation started as early as 2 minutes after the sender was moved into place and reached a plateau of $24.7 \pm 2.5\%$ after 10 minutes. Interestingly, the average number of filopodia on a given growth cone was not affected by the presence of the sender ($p = 0.82$) (Fig. 3.2C). Importantly, the effect of the sender was limited to the stimulated growth cone, as stimulation with a B5 sender for 15 minutes induced a significant increase in filopodial length on B5 receiver growth cones compared to other growth cones on the same cell that were located further away from the sender ($p \leq 0.001$) (Fig. 3.2D). These results, obtained with the sender/receiver paradigm, suggested that B5 neurons

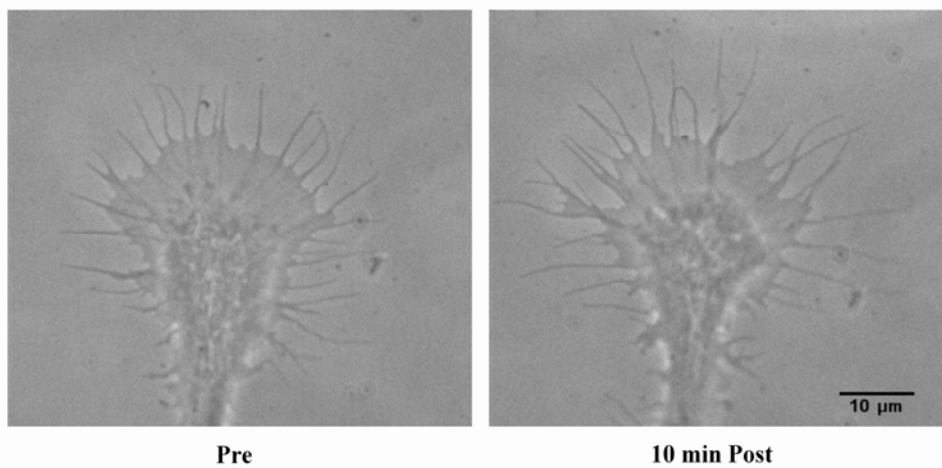
Figure 3.2: Stimulation of B5 receiver growth cones by B5 senders results in an increase in filopodial length.

(A) Phase contrast images of a representative B5 receiver growth cone 5 min before (pre) and 10 min after stimulation by a B5 sender. Note strong filopodial elongation elicited by a B5 soma held approximately 30 μm ahead of the growth cone and located just outside of the image. Scale bar, 10 μm . **(B)** B5 senders held at a distance of 20-30 μm from the growth cone to be tested caused a significant increase in filopodial length on B5 receivers as early as 2 min after placement of the sender and reached a plateau after 10 min when compared to pipette stimulation alone ($p \leq 0.001$). Filopodial length was not affected by the pipette alone. Cell bodies of B19 neurons used as senders had no effect on filopodial length ($p = 0.06$). **(C)** B5 and B19 senders did not cause changes in filopodial number compared with the pipette treatment alone ($p = 0.82$ and $p = 0.83$ respectively). The pipette alone did not change filopodial number. **(D)** A 15 min stimulation with a B5 sender induced a significant increase in filopodial length on B5 receiver growth cones compared to growth cones on the same cell located farther away from the sender ($p \leq 0.001$).

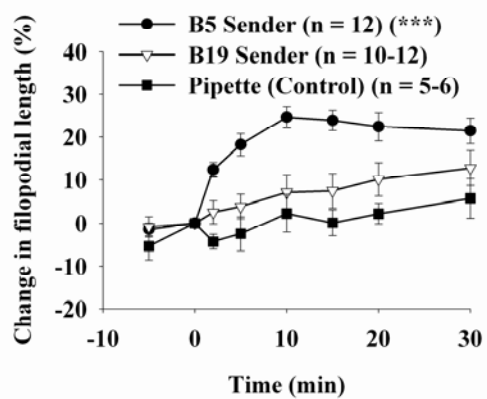
For all experiments the number of growth cones studied per condition is given as 'n'.

Statistical significance between conditions is symbolized by *asterisks* as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

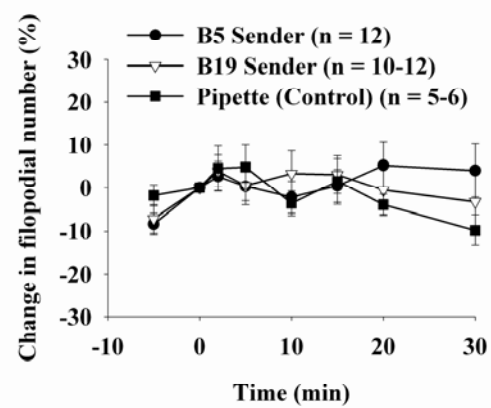
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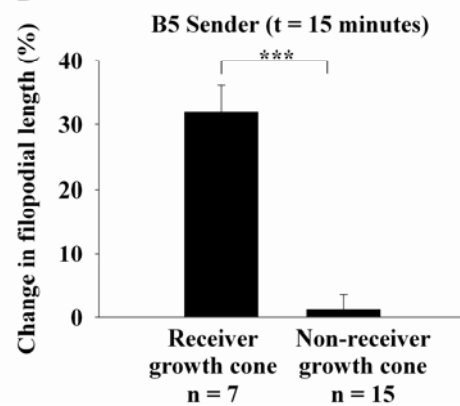
B



C



D



release a diffusible factor(s) that can affect filopodial dynamics on B5 growth cones nearby.

To confirm that the effect of the B5 sender was owing to the release of NO and not some other diffusible factor released by neurons, we next tested the effect of another identified neuron, B19, which does not express nNOS (Van Wagenen and Rehder, 2001). When B19 cell bodies were used as the sender and positioned approximately 20 μm from B5 growth cones, filopodial length and number were not significantly affected ($p = 0.06$ and $p = 0.83$ respectively) when compared to the micropipette alone group, but filopodial length differed significantly from the condition in which a B5 neuron was used as the sender ($p \leq 0.01$) (Fig. 3.2B and C). Not surprisingly, the B19 sender also had no significant effect on filopodial number ($p = 0.99$, as compared to B5 senders) (Fig. 3.2C).

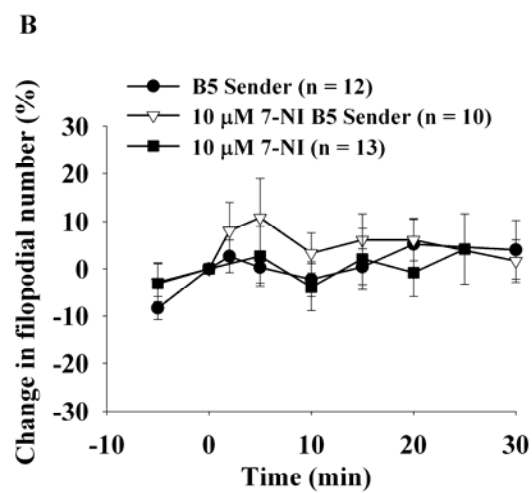
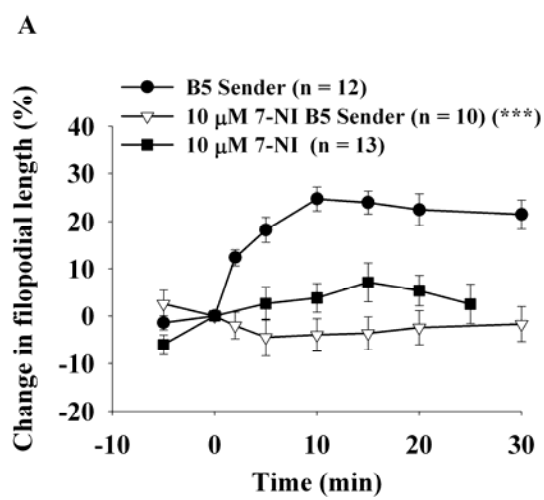
To test independently whether the effects of a B5 sender were mediated by NO, B5 sender/receiver pairs were preincubated with the specific nNOS inhibitor 7-NI (10 μM) for 20 minutes before moving the B5 sender into the proximity of the receiver growth cone. Treatment with 7-NI blocked the filopodial elongation normally induced by B5 senders ($p \leq 0.001$, Fig. 3.3A), and had no effect on filopodial number ($p = 0.18$) (Fig. 3.3A and B). Preincubation with 7-NI by itself had no effect on filopodial dynamics (Fig. 3.3A and B). Taken together, these results support the hypothesis that B5 senders affect growth cone morphology of neurons in their vicinity by releasing NO.

sGC mediates the response of growth cones to NO released by a physiological source

The NO signaling pathway has been shown to act mainly through activation of soluble guanylyl cyclase (sGC) (Arnold et al., 1977; Ahern et al., 2002), and direct S-nitrosylation of proteins (Jaffrey et al., 2001). Because we had shown previously that NO donors induce changes

Figure 3.3: Inhibition of nNOS prevents the B5 sender-induced increase in filopodial length.

(A) Pretreatment with the nNOS inhibitor 7-Nitroindazole (7-NI) (10 μ M) for 20 min abolished the increase in filopodial length induced by B5 senders ($p \leq 0.001$ compared with B5 senders alone). 7-NI itself had no effect on filopodial length. **(B)** Pretreatment with 10 μ M 7-NI followed by stimulation with B5 senders did not change filopodial number ($p = 0.18$ compared with B5 senders alone). Filopodial number was not affected by 7-NI itself. Note that the data for the B5 sender from Fig. 3.1B and C have been added to facilitate comparison.



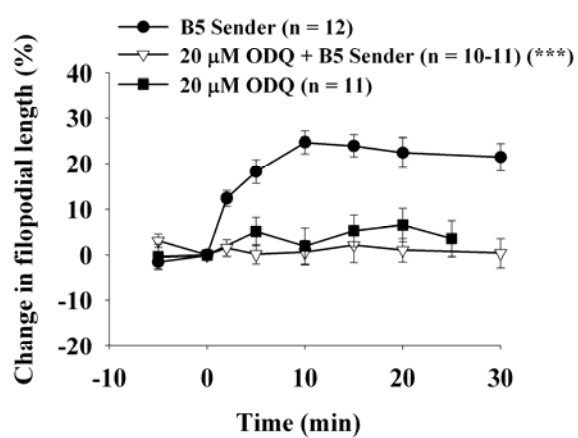
in growth cone morphology of B5 neurons through activation of sGC (Van Wagenen and Rehder, 1999), it was important to confirm that NO released from a physiological source in the sender/receiver paradigm would act through the same signaling pathway. To this end, B5 sender/receiver pairs were preincubated with the sGC inhibitor ODQ (20 μ M) for 20 minutes before moving the sender into the proximity of the receiver growth cone. Treatment with ODQ blocked changes in filopodial length normally induced by B5 senders ($p \leq 0.001$) (Fig. 3.4A). Again, filopodial number was not affected by this experiment ($p = 0.17$) (Fig. 3.4B). The incubation with ODQ alone did not affect growth cone morphology (Fig. 3.4A and B). These results are consistent with our previous findings and suggest that the NO released from a physiological source acts via its canonical target, sGC, to regulate filopodial length.

The effect of NO released from a physiological source is reversible and distance- dependent

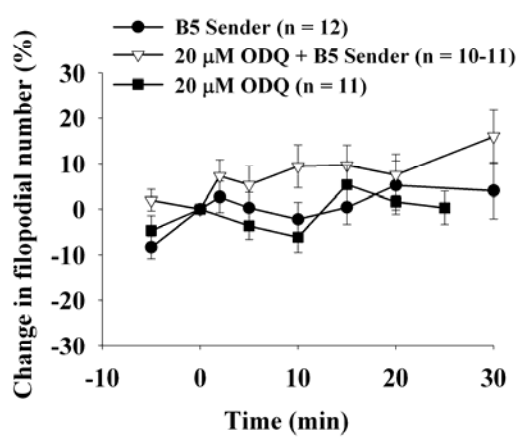
Previous studies from our laboratory showed that application of the NO donor SIN-1 to an entire cell culture dish (global stimulation) induced a rapid increase in filopodial length which showed a transient peak and a subsequent sustained plateau (Van Wagenen and Rehder, 1999; Van Wagenen and Rehder, 2001). By contrast, short-term stimulation with the NO donor NOC-7 applied locally to a growth cone via a micropipette for about 40 ms caused only transient filopodial elongation (Welshhans and Rehder, 2005), suggesting that the NO effect is dependent on the duration of the stimulation with NO. This hypothesis was supported further by the findings in this paper, in which we demonstrate that filopodia remained elongated in the presence of the B5 sender (Fig. 3.2B). Therefore, we next investigated whether the effect of NO on filopodial length was reversible. To address that question, B5 senders were maneuvered into the proximity of B5 receivers (20-30 μ m) for 15 minutes, then the senders were subsequently moved away and filopodial

Figure 3.4: Inhibition of sGC abolishes the B5 sender-induced increase in filopodial length. (A) Pretreatment with the sGC inhibitor 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (20 μ M) for 20 min prevented the increase in filopodial length induced by B5 senders ($p \leq 0.001$ compared with B5 senders alone). ODQ itself had no effect on filopodial length. (B) Pretreatment with 20 μ M ODQ followed by stimulation with B5 senders did not result in a change in filopodial number ($p = 0.17$ compared with B5 senders alone). Filopodial number was not affected by ODQ itself. Note that the data for the B5 sender from Fig. 3.1B and C has been added to facilitate comparison.

A



B



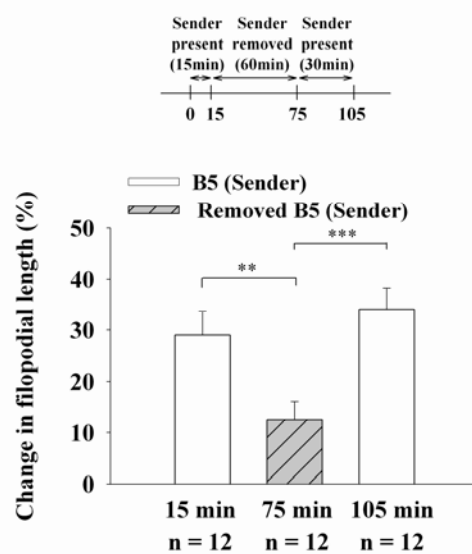
length re-assessed one hour later. Whereas filopodial length increased by $28.9 \pm 4.7\%$ after 15 minutes of pairing with a B5 sender, subsequent removal of the sender resulted in a significant decrease in filopodial length toward baseline, reaching $12.5 \pm 3.5\%$ above pretreatment levels after 1 hour ($p \leq 0.01$) (Fig. 3.5A). It is interesting to note that filopodial length did not return to baseline even after 2 hours of sender removal (data not shown), suggesting that the exposure to NO had resulted in a long term change in the receiver growth cone. To test if a growth cone would be responsive to a second exposure of NO, a B5 sender was brought in again after one hour and positioned 20 μm away from a receiver growth cone. This second exposure to a sender induced another significant increase in filopodial length from the previously elevated plateau of $12.5 \pm 3.5\%$ to $34 \pm 4.3\%$ after 30 minutes ($p \leq 0.001$) (Fig. 3.5A). It is interesting to note that there was no significant difference between the degree of filopodial elongation induced by the first and second presentation of the sender ($p = 0.283$). These results suggested that the effect of NO release from a physiological source, while being partially reversible, has a long lasting effect on the receiver. Nevertheless, growth cones were capable of responding with filopodial elongation to a second exposure to a B5 sender, suggesting that they had maintained their sensitivity to NO.

Next we investigated the distance over which a sender could affect the morphology of a receiver growth cone. To this end, we systematically increased the distance between the sender and the receiver. There was no significant difference in the degree of filopodial elongation when a B5 sender was placed at a distance of either 20-30 μm or 60 μm ($p = 0.334$) from a receiver growth cone ($23.9 \pm 2.4\%$ and $26.3 \pm 3.4\%$ respectively after 15 minutes (Fig. 3.5B). When the B5 sender was located at a distance of 100 μm , filopodial elongation was significantly reduced, to $11.9 \pm 2.1\%$ ($p \leq 0.05$ and $p \leq 0.001$ compared to 20-30 μm and the control (pipette) respectively) (Fig. 3.5B). When the distance between sender and receiver was increased further to 150 μm , the sender no longer had

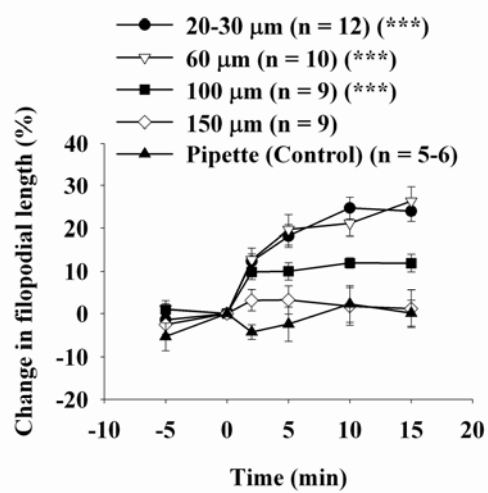
Figure 3.5: A B5 sender can act over a distance of at least 100 μm and its effect on filopodial length is partially reversible.

(A) Removal of B5 senders from B5 receiver growth cones for 60 min resulted in a significant reduction in filopodial length on growth cones stimulated previously by a sender for 15 min ($p \leq 0.01$). Growth cones remained responsive to NO after sender removal, because moving a sender back into the proximity of a receiver growth cone for 30 min (also see above) resulted in another significant increase in filopodial length ($p \leq 0.001$). **(B)** Stimulation with B5 senders held to a distance of 60 μm induced the same degree of filopodial elongation as senders held at 20-30 μm ($p = 0.334$). Senders held at 100 μm induced filopodial elongation ($p \leq 0.001$ compared with pipette), but the elongation was significantly less than the one induced by senders held at 20-30 μm ($p \leq 0.05$). Senders held at 150 μm had no effect on filopodial length ($p = 0.184$).

A



B



a measurable effect on the receiver ($p = 0.184$). This series of experiments suggested that the effective distance over which NO can have a physiological effect on filopodial dynamics in this *in vitro* preparation is at least 100 μm .

Discussion

In the nervous system, nitric oxide has been shown to be involved in numerous processes, including development, synaptic plasticity, and cell death (Ernst et al., 1999; Lipton, 1999; Thippeswamy et al., 2001; Contestabile and Ciani, 2004; Regulski et al., 2004; Matarredona et al., 2005; Bicker, 2007; Hara and Snyder, 2007; McFarland et al., 2007). NO is produced enzymatically by nitric oxide synthase (NOS), which has 3 isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) (Garthwaite, 1991). NO is known to act by several mechanisms, including the activation of the enzyme soluble guanylyl cyclase (sGC) by the binding of NO to its heme moiety (Dierks and Burstyn, 1996), nitrosylation of cysteine residues in proteins to produce S-Nitrosothiols (Arnold et al., 1977; Stamler et al., 1992; Jaffrey et al., 2001; Ahern et al., 2002), and nitration of tyrosine residues (Ischiropoulos, 1998). In addition to its effect on protein regulation, NO can also act on DNA (Wink et al., 1991).

During nervous system development and regeneration, neuronal pathfinding describes the process by which growth cones navigate to their targets, using diffusible and membrane bound cues encountered along the way. There are many guidance cues, such as netrins, semaphorins, ephrins and cell adhesion molecules, which are known to signal to the passing growth cone and potentially affect the speed and direction of growth cone advance (Tessier-Lavigne and Goodman, 1996; Dickson, 2002; Chilton, 2006), however the potential of the gaseous messenger NO to affect neuronal pathfinding and cell motility is only beginning to be explored. Early

studies used various NO donors to test the effect of NO on growth cone motility, neurite outgrowth and neurite initiation (Hindley et al., 1997; Van Wagenen and Rehder, 1999; Rialas et al., 2000; Gallo et al., 2002; Gendron et al., 2002; Trimm and Rehder, 2004; Zhang et al., 2005; Yamada et al., 2006). We have shown previously that global application of nitric oxide (NO) with NO donors can induce changes in both growth cone dynamics and neurite outgrowth in an identified neuron from the buccal ganglion of the mollusk *Helisoma trivolvis*. In the B5 neuron, NO induces an increase in filopodial length, a decrease in filopodial number and a decrease in neurite outgrowth (Van Wagenen and Rehder, 1999; Trimm and Rehder, 2004). Moreover, we have demonstrated that the increase in filopodial length is accomplished by an increase in both the velocity with which filopodia extend and the time they spend extending (Van Wagenen et al., 1999). The reduction in the number of filopodia could be explained by a decrease in the rate with which new filopodia are inserted into the lamellipodial veil to make up for those filopodia being reabsorbed. In addition we have shown that NO induces the same changes in growth cone morphology when it is applied locally and only for a brief time to a single growth cone, a stimulus that potentially more closely mimics a physiological condition (Welshhans and Rehder, 2005).

Whereas the NO donors used in the experiments described above have different half times of release, which make them useful tools for testing effects of NO, a more physiologically relevant description of the effects of NO on the nervous system will have to include experiments in which the effects of cellular release of NO on cellular targets are studied. Studies in the grasshopper reported a role for NO in regulating neuronal migration in the enteric nervous system and axonal pathfinding of pioneer neurons in the antenna (Seidel and Bicker, 2000; Haase and Bicker, 2003; Bicker, 2007). While these studies clearly demonstrate that cell

migration is controlled by cells releasing NO along the path of migratory cells, a direct effect of cellular NO release on growth cone motility has yet to be demonstrated.

Here we use a sender/receiver paradigm to study the effect of cellular NO release on growth cone motility. A cell that releases NO constitutively is used as the physiological NO source and functions as the sender which can be moved easily to any location within the dish. This approach is more convenient than plating two cells close to each other, in the hopes that their processes will grow towards each other. While the latter approach makes it difficult to control for the exact distance between sender and receiver, the movable sender paradigm increases the experimenter's control over the experimental design.

NO released from a physiological source affects growth cone dynamics via sGC

Stimulation of B5 receiver growth cones by B5 sender cell bodies induced a strong and rapid increase in filopodial length (Fig. 3.2A and B). Interestingly this effect on filopodial dynamics was abolished by inhibition of NOS with the specific NOS inhibitor 7-NI (10 μ M). Moreover, stimulation with B19 neurons (Fig. 3.2B), which were shown previously to contain little or no NOS immunoreactivity (Van Wagenen and Rehder 2001) had no effect on filopodia of B5 receivers, further supporting the hypothesis that filopodial elongation was NO-dependent. Other examples of an effect of neuronal NO release on neighboring cells exist, suggesting that release from one cell can affect the physiology of a neighboring cell. For example, electrical stimulation of B2 neurons in another mollusk, *Lymnaea stagnalis*, induces depolarization of B7nor neurons via signaling by NO in both the intact central nervous system and in cell culture (Park et al., 1998). In the hippocampus, evidence has been provided for NO to act as the elusive retrograde messenger in synaptic plasticity, namely long-term potentiation (Bohme et al., 1991;

O'Dell et al., 1991; Schuman and Madison, 1991; Bon and Garthwaite, 2001; Bon and Garthwaite, 2003; Hopper and Garthwaite, 2006). There is a convincing literature that neurotransmitters can affect neurite outgrowth (McCobb et al., 1988; Lipton and Kater, 1989; Spencer et al., 2000; van Kesteren and Spencer, 2003; Fricker et al., 2005; Homma et al., 2006; Ryan et al., 2007), and a sender/receiver paradigm similar to the one used in our study was employed with *Lymnaea* neurons to demonstrate that dopamine can affect growth cone motility. In this study, dopamine released from the right pedal dorsal 1 (RPeD1) neuron was shown to regulate growth cone behavior of target and non-target neurons differentially (Spencer et al., 2000). Here we demonstrate that NO, released by a single cell, can have a strong effect on growth cones in its vicinity but not on growth cones located further away, clearly demonstrating that NO has the potential to affect neurite outgrowth locally. Our results also confirm earlier reports on the same cell type that demonstrated that NO brought about its effect on growth cone morphology and advance via activation of sGC (Van Wagenen and Rehder, 1999; Trimm and Rehder, 2004), as the effect of the sender on growth dynamics of the receiver was blocked in the presence of the sGC inhibitor ODQ (Fig. 3.4).

The effect of cellular NO release on filopodial dynamics is reversible and dose dependent

Whereas previous studies *in vivo* have demonstrated effects of NO on cell motility and neurite outgrowth (Gibbs and Truman, 1998; Chen et al., 2000; Seidel and Bicker, 2000; Haase and Bicker, 2003; Bicker, 2005), these experiments were mainly based on pharmacology to inhibit NO signaling and their conclusions were, therefore, somewhat indirect. Our results suggest that B5 neurons produce NO constitutively and that a growth cone entering the “plume” of NO created by such a sender cell would respond by elongating its filopodia. This is the first

report in which the effects of NO released from a single cell have been measured using growth cone motility as a sensitive bioassay. Our results clearly demonstrate that a single, unstimulated cell body can release enough NO to affect an advancing growth cone. Because NOS, in the case of the B5 neuron, is expressed throughout the entire neuron, we envision that even growth cones of B5 neurons can release NO, possibly affecting other cells in their vicinity. Clearly, the B5 neuron may be unique in that it produces NO and also responds to it. As such, it could serve as a source of NO for its own purposes as well as supplying NO to the larger environment. We have shown previously that B5 neurons are responsive to the NO they produce constitutively, as neurite outgrowth slowed when either NOS or sGC was inhibited, suggesting that intrinsically produced NO plays a role in determining the speed of neurite advance (Trimm and Rehder, 2004). It is reasonable to assume that other cells in the snail CNS will show a more conventional separation between NO producing and sGC expressing cells, making NO a paracrine modulator. Because the NOS enzyme is activated via Ca/calmodulin, it is likely that the amount of NO being produced and released by a B5 neuron will depend on the intracellular calcium concentration, and would thus be modulated by neuronal activity. Future studies will investigate the relationship of cellular electrical activity and NO release.

Interestingly, the effect of NO release on filopodial length was reversible, as filopodia shortened again after the NO sender was removed, but the reversibility was incomplete, indicating that some long-term changes in the state of the growth cone had resulted from a 15 minute exposure to NO. Additionally, it is expected that any effect of NO would be dose dependent. Modeling studies of NO release, taking into account the half life and diffusion of NO from a point source, suggest that NO might have physiological effects in a 200 μm diameter sphere (Lancaster, 1994; Wood and Garthwaite, 1994; Lancaster, 1997), but the action radius of

NO will clearly depend on the dynamics of NO production, its decay, and the general cellular environment. Our results show clearly that the effect of NO varied inversely with the distance between the sender and the receiver growth cone. While maximal effect was seen between 20-60 μm , NO still affected growth cones that were located 100 μm away, but failed to elicit an effect at 150 μm . These results fall nicely into the range of effective distances as reported above. It should be kept in mind, however, that our experiments were performed on single cells *in vitro*, and that the NO radius of action in the intact nervous system might well be different.

NO released from tissues and cells within tissues has been detected by NO electrodes (Friedemann et al., 1996; Bedioui et al., 1997) and the use of fluorescent dyes, such as DAF-2A (Kojima et al., 1998), but a quantification of the amount of NO released from single cells is not trivial. Recently, Patel et al. (2006) reported that NO released from single neurons in the isolated CNS of *Lymnaea stagnalis* was detected by multiple film-coated electrode sensors. A cell-based indicator, called Picell, detected picomolar concentrations of NO released from single endothelial cells and hippocampal neurons in culture (Sato et al., 2006). These tools will be important in future investigations to directly measure the spatio-temporal NO release from B5 senders.

A role for NO in neuronal pathfinding and synaptogenesis

Neuronal pathfinding takes place on the background of a rapidly changing nervous system, both molecularly and structurally. Well orchestrated expression of guidance cues and their cellular receptors play an important part in guiding an axon towards its target and assist in synaptogenesis (Martinez et al., 1998; Kaprielian et al., 2001; Martinez et al., 2005; Dickson and Gilestro, 2006; Mendes et al., 2006). We do not know for our system whether NOS or the

presence of its cellular target sGC is developmentally regulated. Studies in the snail *Ilyanassa obsoleta* reported an upregulation of NOS in the developing nervous system during the larval stage, followed by a down regulation through metamorphosis (Lin and Leise, 1996; Thavaradhara and Leise, 2001). The developmental regulation of both NOS and sGC in the enteric nervous system of the locust serve as a impressive example of how differential expression may be useful in establishing the early migratory outcome of cells along the midgut (Haase and Bicker, 2003; Bicker, 2005; Bicker, 2007).

We have presented data that demonstrate that NO donors applied to neurons grown in cell culture can affect growth cone motility and it is reasonable to assume that NO might influence neuronal pathfinding *in vivo*. Our observation that NO resulted in filopodial elongation and a slow-down in growth cone advance, a response we termed ‘slow down and search’ behavior, could indicate a role for NO as a stop signal in the target region prior to synaptogenesis. NOS has been localized in target regions in several systems and its expression correlated with synaptogenesis (Roskams et al., 1994; Williams et al., 1994; Ogilvie et al., 1995). Recent studies reported that presynaptic plasticity induced during activity-dependent synaptogenesis was regulated by NO (Nikonenko et al., 2003; Zhang et al., 2005). Moreover, there is evidence that NO regulates the activity dependent refinement of retinal projections. Inhibition of nNOS by pharmacological blockers was shown to prevent the elimination of transient ipsilateral retinotectal projections in the chick visual system, and to affect retinogeniculate projection patterns in ferret (Wu et al., 1994; Cramer and Sur, 1999).

In conclusion, we have demonstrated that spontaneous NO release from a single neuron has physiological effects on the dynamics of growth cones growing in its vicinity, and that this effect is mediated through the canonical target of NO, namely sGC. These results strongly

suggest a potential role for NO as a modulator of growth cone motility during neuronal pathfinding and synaptogenesis *in vivo*.

Chapter 4 : Research Published in Collaboration: PLA₂ and Secondary Metabolites of Arachidonic Acid Control Filopodial Behavior in Neuronal Growth Cones

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K.Tornieri contributed to the writing of the manuscript and to Figures 4 and 7 in the original publication.

Abstract

The neuronal growth cone provides the sensory and motor structure that guides neuronal processes to their target. The ability of a growth cone to navigate correctly depends on its filopodia, which sample the environment by continually extending and retracting as the growth cone advances. Several second messengers systems that are activated upon contact with extracellular cues have been reported to affect growth cone morphology by changing the length and the number of filopodia. Because recent studies have suggested that guidance cues can signal via G-protein coupled receptors to regulate phospholipases, we here investigated whether phospholipase A₂ (PLA₂) may control filopodial dynamics and could thereby affect neuronal pathfinding. Employing identified *Helisoma* neurons *in vitro* we demonstrate that inhibition of PLA₂ with 2 μ M BPB caused a 40.3% increase in average filopodial length, as well as a 37.3% reduction in the number of filopodia on a growth cone. The effect of PLA₂ inhibition on filopodial length was mimicked by the inhibition of G-proteins with 500 ng/ml pertussis toxin and was partially blocked by the simultaneous activation of PLA₂ with 50nM melittin. We provide evidence that PLA₂ acts via production of arachidonic acid (AA), because (a) the effect of inhibition of PLA₂ could be counteracted by supplying AA exogenously, and (b) the inhibition of cyclooxygenase, which metabolizes AA into prostaglandins also increased filopodial length. We conclude that filopodial contact with extracellular signals that alter the activity of PLA₂ can control growth cone morphology and may affect neuronal pathfinding by regulating the sensory radius of navigating growth cones.

Introduction

Phospholipase A₂ (PLA₂) is a group of enzymes that catalyze the breakdown of phospholipids. They are activated by signaling through calcium (van den Bosch, 1980), kinases (Lin et al., 1993), growth factors (Spaargaren et al., 1992), or through metabotropic receptors, such as glutamate receptors (Dumuis et al., 1993). The mechanism by which G-proteins activate PLA₂ is unclear, as activation has been reported to occur through an unknown G-protein (Tsunoda and Owyang, 1994) or the activity of G_i (Dickerson and Weiss, 1995). While PLA₂ plays a role in membrane maintenance and breakdown (Bonventre, 1992), it also produces a number of active messengers with wide ranging cellular functions (Axelrod, 1990). PLA₂ cleaves phospholipids, thereby producing arachidonic acid (AA) and lysophospholipids (Negre-Aminou and Pfenninger, 1993). Messengers produced by PLA₂ activity have been demonstrated to affect neurite advance, as inhibition of PLA₂ decreases outgrowth in dorsal root ganglion neurons from the adult frog (Edstrom et al., 1996) and mouse (Hornfelt et al., 1999). In addition, PLA₂ is localized to rat nerve growth cones (Negre-Aminou et al., 1996) and is necessary for growth cone formation (Geddis and Rehder, 2003). Finally, PLA₂ is involved in thrombin-induced (de La Houssaye et al., 1999) and semaphorin 3A-induced (Mikule et al., 2002) growth cone collapse. Taken together, the involvement of PLA₂ in neurite outgrowth and its localization to the growth cone suggest that it may play an important role in generating growth cone behavior.

In the developing nervous system, neuronal growth cones navigate through a complex environment in order to make appropriate synaptic connections. Filopodia have been shown to be of critical importance in this process, as growth cones stripped of their filopodia are impaired in their pathfinding abilities (Bentley and Toroian-Raymond, 1986). Receptors on filopodia contact cues ahead of the growth cone proper, and the ability of filopodia to undergo length changes

provides a growth cone with the capability to adjust its action radius according to the particular pathfinding situation. The detection of extracellular cues by receptors on filopodia is thought to activate intracellular signals that control growth cone behavior such as growth cone advance (Nakamura et al., 2000) and steering (O'Connor et al., 1990). It has been reported previously that intracellular signaling molecules such as phosphatases, kinases and calcium are involved in controlling growth cone behavior in identified *Helisoma* neurons (Davenport and Kater, 1992; Rehder and Kater, 1992; Welnhofer et al., 1999). Whereas our knowledge of the signaling mechanisms downstream of extracellular ligands such as semaphorins, netrins and certain growth factors (such as EGF and NGF) is increasing rapidly (Handler et al., 1990; Weisenhorn et al., 1999; Segal, 2003), we know very little about the potential role of lipid-derived messengers in regulating growth cone behavior.

Given the central role played by filopodia in growth cone navigation, this study investigates mechanisms by which phospholipase A₂-mediated second messengers control filopodial dynamics. A pharmacological approach is employed to demonstrate that PLA₂ activity regulates filopodial length and number through the production of arachidonic acid and two of its products, prostaglandins and leukotrienes. Therefore, guidance cues which signal through PLA₂ should be considered as potential candidates to direct the outcome of neuronal pathfinding.

Methods and Materials

Neuronal culture and imaging

Identified B5 neurons were dissected from *Helisoma trivolvis* buccal ganglia and plated for neuronal culture. Neurons were plated onto a poly-L-lysine-(hydrobromide, MW 70,000-150,000, 0.25 mg/ml, Sigma, St. Louis, MO) coated glass coverslip glued to the bottom of a 35

mm cell culture dish. Cells were allowed to grow overnight at room temperature and used 24-48 hours after plating. Cells were grown in conditioned medium prepared by incubating snail brains in Leibowitz L-15 medium (special order, Gibco, Grand Island, NY) modified to a final concentration of: 40 mM NaCl, 1.7 mM KCl, 1.5 mM MgCl₂, 4.1 mM CaCl₂, 10 mM HEPES, 50 μ g gentamycin/ml, 0.15 mg glutamate/ml in distilled water (pH = 7.3). Growth cones were viewed under a 100x oil immersion objective on an Axiovert (Carl Zeiss, Thronwood, NY) or a Sedival (aus Jena, Germany) microscope. Images were captured with a cooled CCD (CH250, Photometrics, Tucson AZ) or C-72 camera (MIT Dage, Michigan City, IN) interfaced with NIH image software (Wayne Rasband, NIH). The images were viewed and analyzed on a Power Macintosh (Apple Computer, Cupertino, CA).

Pharmacological treatments

Observation of growth cone behavior, specifically changes in filopodial length and number, was performed at defined times before and after the addition of pharmacological agents, which were added through a complete medium exchange. Images were captured before (-5 and 0 min) and at defined times after (2, 5, 10, 15, 20, 30 minutes) a complete medium exchange, which introduced the experimental condition. A complete medium exchange was accomplished by removing all medium from the dish, except from the center well formed by the coverslip attached to the dish (approximate volume: 200 μ l), and by adding 2 ml of L-15 medium containing the premixed drug. The phospholipase 2A (PLA₂) inhibitors, 4-bromophenacyl-bromide (BPB) was obtained from Sigma and dissolved to stock concentrations of 2.5 mM in warm methanol. All other drugs were purchased from Calbiochem (La Jolla, CA). The cyclooxygenase (COX-2) inhibitor, diclofenac sodium{2-[(2',6'-Dichlorophenyl)amino] benzeneacetic Acid, Na}

was dissolved in water to a stock concentration of 5 mM. Cytochalasin B (*Helminthosporium dematioideum*) was dissolved in DMSO to a stock concentration of 4 mM, and arachidonic acid (porcine liver, sodium salt) was dissolved in water to a stock concentration of 200 mM. Control experiments were performed by addition of the solvents used for the reconstitution of the pharmacological agents above.

Data analysis and statistics

Growth cone behavior was assessed by determining the change in filopodial length and number as previously described (Van Wagenen and Rehder, 1999). By using fiduciary markers present in the dish, filopodial elongation was measured as active protrusion of filopodial tips and thus ruled out “apparent” elongation, which can result from lamellipodial retraction. Values are expressed as mean \pm SEM. Significance between conditions was determined by performing an analysis of variance (1-way ANOVA or ANOVA with time as repeated measure) with SuperANOVA (Abacus Concepts, Berkeley, CA). Fisher’s Protected Least Significant Difference (PLSD) test was used for post-hoc tests of statistical significance. Significant differences are indicated as: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Results

Filopodial length is controlled by PLA₂ activity through arachidonic acid and its secondary metabolites

To examine how phospholipase 2A (PLA₂) may affect filopodial length, and by extension, the action radius of a navigating growth cone, the activity of PLA₂ was inhibited by

the addition of the specific PLA₂ inhibitor 4-bromophenacyl-bromide (BPB). 2 μ M BPB induced an increase in filopodial length (Fig. 4.1). To investigate further the pathway by which PLA₂ mediated its effect on filopodial behavior, we examined first the role of AA, the major product of PLA₂ activity, and then key enzymes metabolizing AA (Fig. 4.2). If PLA₂ acted through AA, then it should be possible to interfere with the effect of PLA₂ inhibition by providing AA through alternative sources. AA can be taken up by cells grown in cell culture when added to the extracellular medium (Piomelli et al., 1987; Carlson and Levitan, 1990). Extracellularly applied AA (5 μ M) had no significant effect on filopodial length when added by itself (Fig. 4.2), but it significantly reduced the effect of BPB (2 μ M) on filopodial length when BPB was applied after cells had been preincubated with AA (5 μ M) for 5 minutes ($p \leq 0.05$), supporting the hypothesis that PLA₂ acted via AA. AA is the substrate of two enzymes that produce different eicosanoid messengers (Funk, 2001). AA is converted into leukotrienes by the action of lipoxygenase and into prostaglandins by cyclooxygenase (Fig. 4.2). If PLA₂ acted through one of these pathways, inhibition of these pathways should mimic the inhibition of PLA₂ itself. Indeed, inhibition of cyclooxygenase with diclofenac sodium (100 μ M) caused filopodial elongation (Fig. 4.3B). Quantification of this effect showed that filopodial length was elevated by $28.3 \pm 3.1\%$ as early as five minutes after cyclooxygenase inhibition.

PLA₂ and cyclooxygenase affect filopodial dynamics

Because filopodia are composed of bundled f-actin (Gordon-Weeks, 1987), and filopodial length changes are to a large part dependent on actin polymerization and depolymerization events, we started to investigate whether PLA₂ inhibition might affect actin dynamics and thereby lead to filopodial elongation. To address the question whether filopodial elongation was

Figure 4.1: The effect of inhibition of PLA₂ on filopodial behavior is mediated through arachidonic acid.

Inhibition of PLA₂ with BPB (2 μ M) elicited filopodial elongation of $28.6 \pm 4.3\%$ five minutes after treatment, and this effect was significantly blocked to $15.6 \pm 3.5\%$ when neurons were pretreated for 5 minutes with 5 μ M AA added to the medium ($p \leq 0.05$). The same concentration of AA added alone had no significant effect on filopodial length.

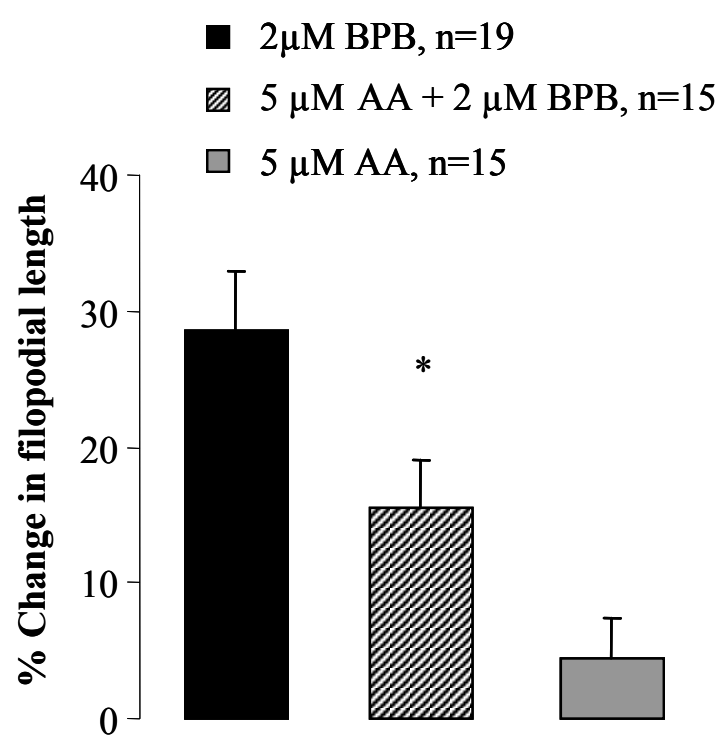


Figure 4.2: Mechanisms of PLA₂ activation and pathways downstream of PLA₂ involved in controlling filopodial dynamics.

Activation of PLA₂ can occur through G-protein coupled receptors or an increase in $[Ca^{2+}]_i$. PLA₂ cleaves phospholipids to produce arachidonic acid (AA) which, in turn, is converted into prostaglandins and leukotrienes by cyclooxygenase and lipoxygenase, respectively. Inhibition of PLA₂ resulted in distinct changes in growth cone behavior, namely filopodial elongation and a reduction in the number of filopodia. AA and its secondary metabolites control filopodial dynamics through the action of leukotrienes and prostaglandins. The mechanisms by which leukotrienes and prostaglandins affect filopodial dynamics is presently unknown and symbolized by a black box.

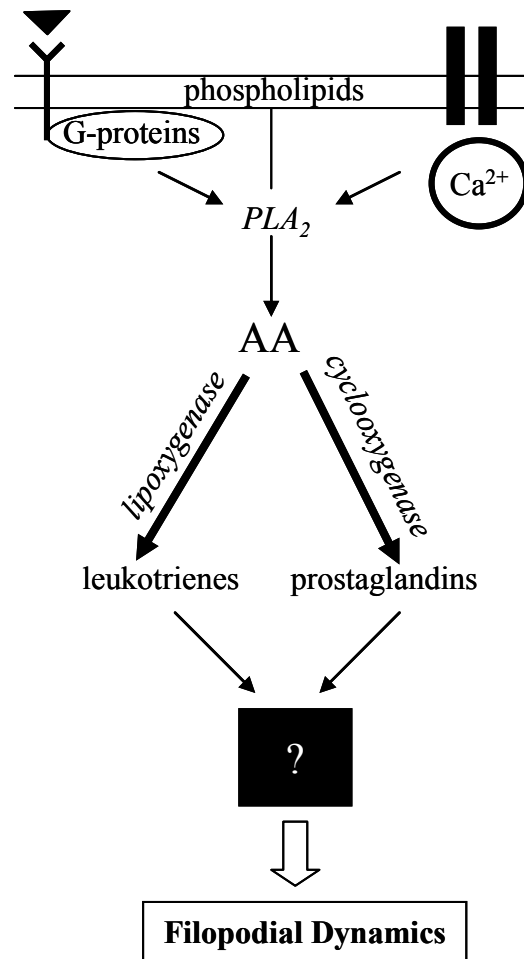
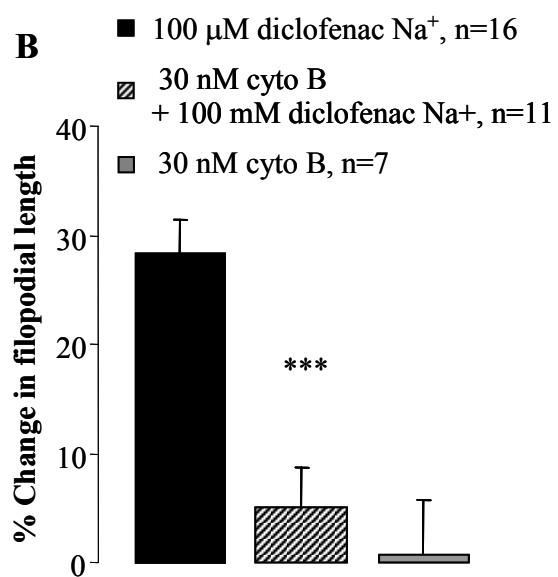
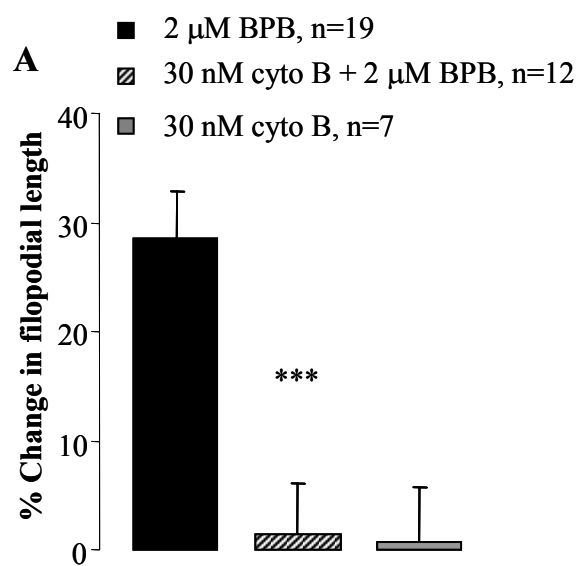


Figure 4.3: Filopodial elongation resulting from inhibition of PLA₂ and cyclooxygenase involves actin polymerization.

(A) The effect of filopodial elongation observed after 5 minutes of inhibition of PLA₂ with BPB (2 μ M) was completely blocked when growth cones were preincubated for 10 minutes with 30 nM cytochalasin B (Cyto B, $p \leq 0.001$). The same concentration of cyto B added alone had no effect on filopodial length. **(B)** Inhibition of cyclooxygenase with diclofenac Na⁺ (100 μ M) resulted in an increase in average filopodial length to $28.3 \pm 3.1\%$ after 5 minutes of treatment. This effect was significantly blocked to $5.1 \pm 3.6\%$ when growth cones were preincubated with 30 nM cyto B for 10 minutes ($p \leq 0.001$) before the addition of diclofenac Na⁺ (100 μ M).



the result of actin polymerization, growth cones were incubated in cytochalasin B (cyto B, 30 nM) for 10 minutes before adding BPB (2 μ M). Cytochalasin inhibits actin monomer addition to the plus end of microfilaments and at this low concentration had no effect on filopodial length (Fig. 4.3A). When BPB was added in the presence of cyto B, however, the effect of BPB was completely inhibited ($p \leq 0.001$), suggesting that the BPB-induced filopodial elongation resulted from actin polymerization. We next investigated if the elongation observed upon inhibition of cyclooxygenase with diclofenac sodium was also due to actin polymerization. Whereas diclofenac sodium had its maximal elongation effect as early as 2 minutes after addition, this effect was completely blocked when the drug was added after growth cones had been incubated in cyto B for 10 minutes ($p \leq 0.001$; Fig. 4.3B).

Discussion

Growth cones are guided by receptors that interact with various guidance cues (Tessier-Lavigne and Goodman, 1996). Ligand-receptor interactions activate particular second messenger systems that cause directed changes in the growth cone's cytoskeleton (Gallo and Letourneau, 2000), which ultimately translate into growth cone steering events. Although there is a considerable body of knowledge describing the role of kinases on growth cone behavior (Jian et al., 1994; Bonsall and Rehder, 1999; Cheng et al., 2000) and neuronal guidance (Jian et al., 1994; Burden-Gulley et al., 1997; O'Leary and Wilkinson, 1999; Wills et al., 2002), we know very little about the potential role of lipid-derived messengers in regulating growth cone behavior. Lipid-derived messengers can be activated by extracellular ligands through various G-protein-coupled receptors (Rozengurt, 1991; Roberts, 1996; Funk, 2001), but their effects on growth cone motility and subsequent neuronal pathfinding have not been well studied. We here

describe the effects of a prominent class of phospholipases, PLA₂, on the regulation of growth cone filopodia. The data presented suggest that the activity of PLA₂ and its downstream products affect growth cone behavior and could function in the signal transduction cascade from extracellular guidance cues to the actin-based cytoskeleton.

Inhibition of PLA₂ causes filopodial elongation and a reduction in filopodial number

In the presence of the PLA₂ inhibitor BPB, the mean filopodial length on growth cones increased rapidly and filopodia remained elongated for at least 30 minutes. The long-term effects on filopodia seen with PLA₂ inhibition may be due to the fact that BPB is known to bind PLA₂ irreversibly (Roberts et al., 1977). Under physiological circumstances, a transient inhibition of PLA₂ activity would be expected to cause short-term filopodial elongation. Such a response would result in a rapid and transient increase in the action radius of a growth cone and allow it to survey a larger area during pathfinding.

Filopodia contain bundled f-actin and theoretically, filopodial length can be controlled by actin polymerization and depolymerization rates, as well as by altering the rate of retrograde actin flow. The experiments in which actin polymerization was inhibited by cytochalasin suggested that filopodial elongation observed under PLA₂ inhibition resulted from actin polymerization. After approximately 10 minutes of PLA₂ inhibition, filopodia stopped elongating. This second, longer-term effect of PLA₂ inhibition, at which filopodia appeared somewhat “frozen”, suggested that eventually the mechanism(s) that result(s) in further filopodial elongation were both inhibited. Our results are consistent with the interpretation that PLA₂ activity is necessary for both filopodial extension and retraction to occur. The notion that PLA₂ activity might be required for actin depolymerization is supported by the result that PLA₂

is necessary for thrombin-induced growth cone collapse (de La Houssaye et al., 1999). Because our experimental approach did not allow us to differentiate fully between effects of PLA₂ on retrograde flow, or on the barbed and pointed ends of f-actin, future studies will be needed to determine which of these mechanisms, or which combination of mechanisms, is responsible for the filopodial elongation observed in these experiments.

Inhibiting the production of AA and its secondary metabolites alters actin dynamics

Our finding that the extracellular addition of AA was able to decrease the effect of PLA₂ inhibition and partially blocked filopodial elongation, suggested that PLA₂ indeed acted through AA. AA has been shown to be taken up by cells (Carlson and Levitan, 1990; Piomelli et al., 1987) and it has to be assumed that the external application of AA increased the intracellular [AA] such that the inhibition of PLA₂, resulting in a reduction of AA production from phospholipids, had a lesser effect on the overall concentration of AA in the cell. Our attempts to fully block the effect of PLA₂ inhibition by providing extracellular AA was unsuccessful, because higher concentrations of AA applied externally caused filopodial lengthening itself (data not shown), potentially due to its effect of raising $[Ca^{2+}]_i$ as has been demonstrated for AA in other cell types (Williams et al., 1994; Roudbaraki et al., 1995).

It has been reported that the products of cyclooxygenase and lipoxygenase activity, prostaglandins and leukotrienes, work to control actin dynamics. For example, in fibroblasts, cyclooxygenase inhibitors increase actin polymerization, whereas lipoxygenase inhibitors are reported to decrease actin polymerization (Peppelenbosch et al., 1993). The former effect is consistent with our results in which the inhibition of prostaglandin production induced filopodial elongation. As suggested by its blockage in the presence of cytochalasin, this filopodial

elongation resulted from actin polymerization and suggested that PLA₂ could control actin dynamics via AA production in *Helisoma* neurons as well. Therefore, the role of PLA₂ in mediating growth cone behavior may be to produce AA that is further metabolized into leukotrienes and prostaglandins, which in turn potentiate the cell's ability to extend and retract its filopodia through actin polymerization/ depolymerization events.

A role for phospholipases in neuronal pathfinding?

Elucidation of the signaling pathways involved in controlling filopodial dynamics will lead to a better understanding of how neuronal growth cones traverse the terrains encountered during pathfinding. In this study we have used a pharmacological approach to demonstrate that the PLA₂ signaling pathway in growth cones affects filopodial behavior by altering filopodial length. To our knowledge, this study is the first to implicate PLA₂ and the secondary metabolites of AA in the process of filopodial regulation. Whereas the effects described here occurred on a time scale of minutes, taken together with findings by others that PLA₂ inhibition reduces the rate of neurite outgrowth on a longer time scale, a picture emerges that PLA₂ inhibition may signal the transformation of a growing growth cone into one that extends its filopodia and then slows down to initiate a searching behavior, as is seen in growth cones that undergo pathfinding decision *in vivo*. We restricted our pharmacological experiments to 30 minutes, because we assume that signaling events mediated through the activation or inhibition of second messenger pathways are likely to be most effective when they are of short duration, and because effects arising from long term stimulation are inherently more difficult to interpret. Taken together, we propose that PLA₂ may be activated by G-protein coupled receptors or by Ca²⁺ to produce AA. AA is then metabolized into leukotrienes and prostaglandins, which act to alter the polymerization and

depolymerization state of actin (Fig. 4.1). Future studies of guidance cues and their activation of phospholipases, such as PLA₂, in migrating growth cones should shed light on the physiological role of PLA₂ in neuronal pathfinding, as well as further clarify the signaling mechanisms that regulate the underlying actin dynamics.

Chapter 5 : General Discussion and Conclusions

During neuronal pathfinding, growth cones located at the tip of axons and dendrites encounter and respond to diffusible and membrane-bound cues along the path toward their targets. These extracellular cues trigger intracellular mechanisms regulating growth cone cytoskeletal dynamics that are important for correct pathfinding and successful synaptogenesis. Although many molecules and signaling pathways linking guidance cues to changes in growth cone cytoskeletal dynamics have been characterized, the aim of this dissertation is to extend our knowledge of mechanisms controlling growth cone morphology via two main studies: (1) To examine how PI-3K affects growth cone dynamics and the identification of its intracellular signaling pathway, and (2) To examine how neuronal NO release can influence the physiology of other neurons nearby, with a focus on effects of NO release on growth cone dynamics.

Molecules and Mechanisms Controlling Growth Cone Dynamics and Motility

Signaling pathways

Growth cones, defined as both sensory and motor structures, are comprised of a central domain, composed of microtubules and organelles, and lamellipodia and filopodia, which are composed of meshworks and bundles of F-actin filaments, respectively (Smith, 1988; Huber et al., 2003). Filopodia continuously extend and retract to sample and respond to their environment during their journey toward their targets, and by doing so, adjust the area they sense at any given point in time, referred to as the “action radius”. Actin-based filopodial dynamics are critical for correct pathfinding: elimination of actin dynamics and subsequent loss of filopodia from embryonic grasshopper pioneer growth cones by treatment with the actin depolymerizing agent cytochalasin leads to misguidance of pioneer neurites (Bentley and Toroian-Raymond, 1986). Treatment of *Xenopus* retinal growth cone with cytochalasin induces misguidance as well (Chien

et al., 1993). Because filopodia are necessary for correct guidance and therefore the proper wiring of the nervous system, understanding the mechanisms controlling their dynamics is critical. Many molecules and second messengers have been identified, but critical gaps in signaling pathways still remain. The current work contributes to a better understanding of growth cone morphology by studying how a molecule, PI-3K, and its downstream targets (ROCK, Akt and MEK) regulate this process.

Calcium has been demonstrated to be critical for growth cone behavior (Kater et al., 1988; Kater and Mills, 1991; Henley and Poo, 2004). In *Helisoma* growth cones, an increase in the intracellular calcium concentration $[Ca^{2+}]_i$ induces an increase in filopodial length (Davenport and Kater, 1992; Rehder and Kater, 1992). An increase in filopodial length was also observed after global and regional release of calcium, via uncaging, in growth cones (Cheng et al., 2002). Calcium also regulates neurite outgrowth (Nishi and Berg, 1981; Mattson and Kater, 1987) and growth cone steering (Henley and Poo, 2004). Growth cone turning induced by a directed source of a guidance cue is mediated by a local increase in $[Ca^{2+}]_i$ on the side stimulated by the cue (Song and Poo, 1999; Hong et al., 2000; Henley and Poo, 2004). Downstream targets of calcium that regulate growth cone dynamics have also been identified. The effect of an increase in $[Ca^{2+}]_i$ on the *Helisoma* B5 growth cone is mediated by calmodulin and the calcium-dependent phosphatase, calcineurin (Cheng et al., 2002), suggesting that the phosphorylation state of the growth cone regulates filopodial dynamics.

In vivo and *in vitro* studies have demonstrated that kinases and phosphatases regulate growth cone guidance (Elkins et al., 1990; Williams et al., 1994; Callahan et al., 1995; Gallo et al., 1997). Treatment of *Helisoma* B5 growth cones with the general protein kinase inhibitor K252A and specific inhibitors of protein kinase C (PKC) result in an increase in filopodial length

and a decrease in filopodial number (Cheng et al., 2000). Consistent with the above evidence, our results provide new information about another protein kinase, phosphatidylinositol-3-kinase (PI-3K), and its downstream targets as regulators of growth cone dynamics (Tornieri et al., 2006). Because PKC is a downstream target of PI-3K (Krasilnikov, 2000), our finding that PI-3K inhibition induces an increase in filopodial length confirms our previous work suggesting a role of PKC in regulating filopodial behavior (Cheng et al., 2000). This effect of PI-3K regulating growth cone morphology also supports previous studies demonstrating a role of PI-3K in neurite outgrowth (Kita et al., 1998; Ditlevsen et al., 2003). In addition PI-3K has been demonstrated to mediate ephrin A5 and slit-2 induced growth cone collapse in chick retinal ganglion cells (Wong et al., 2004). Recently our PI-3K results were supported by a study that demonstrated that neurotrophins regulate dendritic filopodial dynamics in mouse hippocampal cultures through PI-3K (Luikart et al., 2008). In addition to providing the first demonstration of the regulation of growth cone dynamics by PI-3K, our work also unraveled the mechanisms by which PI-3K might regulate growth cone guidance. We have shown that PI-3K affects filopodial dynamics through its double enzymatic activity, namely its lipid kinase and protein kinase activities. Our finding that inhibition of Rho-activated protein kinase (ROCK) causes filopodial elongation confirms a report on retinal ganglion neurons in which filopodial elongation was elicited by inhibition of the ROCK effector RhoA, and ROCK itself (Gehler et al., 2004; Gehler et al., 2004). **Taken together, our work demonstrates a role for PI-3K kinase in regulating growth cone dynamics during neuronal pathfinding via both its lipid and protein kinase activities.**

Filopodial dynamics

Filopodia are continuously going through cycles of extension and retraction. Thus, overall filopodial elongation can be explained by changes in dynamic parameters, such as the rate at which filopodia extend and retract, as well as the amount of time that an individual filopodium spends extending, retracting or not moving. Our laboratory has previously demonstrated that NO-induced filopodial elongation is achieved by increasing the rate at which filopodia elongate and the time that individual filopodia spend extending (Van Wagenen et al., 1999). The results of changes in filopodial dynamic parameters are supported by our findings that the filopodial elongation observed after inhibition of PI-3K is also mediated by a combination of an increase in the extension rate and the time that filopodia spend extending (Chapter 2). Possible changes in cytoskeletal dynamics explaining these modifications of filopodial dynamic parameters will be explained in detail later in the discussion. It will be of interest in the future to investigate if these changes in filopodial dynamic parameters constitute a general mechanism mediating growth cone filopodial extension or if they are specific to particular signaling pathways.

Physiological effect of NO on growth cone dynamics

The gaseous messenger nitric oxide (NO) has been demonstrated to regulate different processes in the nervous system, including development, synaptic plasticity, cell death and neurogenesis (Ernst et al., 1999; Lipton, 1999; Contestabile and Ciani, 2004; Matarredona et al., 2005; Bicker, 2007). The fact that NO is a gas makes it an unconventional messenger, which can diffuse across cell membranes and affect target molecules in neighboring cells. In the nervous system, NO is formed by the conversion of L-arginine and oxygen to L-citrulline by

calcium/calmodulin-dependent neuronal nitric oxide synthase (nNOS) (Alderton et al., 2001). Previous studies in B5 *Helisoma* neurons demonstrated that global application of NO donors induces an increase in filopodial length and a decrease in neurite outgrowth *in vitro* (Van Wagenen et al., 1999; Trimm and Rehder, 2004), suggesting a role for NO as a guidance cue regulating neuronal pathfinding. This role of NO was further supported by a recent finding that focal application of NO onto a single growth cone induces an increase in filopodial length locally on the stimulated growth cone via soluble guanylyl cyclase (sGC), protein kinase G (PKG), cyclic adenosine diphosphate ribose (cADPR) and intracellular calcium release from ryanodine sensitive stores (Welshhans and Rehder, 2005; Welshhans and Rehder, 2007).

However, this work examines the ultimate demonstration of a role for NO in affecting growth cone motility, by studying the effect of physiological concentrations of NO on growth cone dynamics. Although both NO release from NO-donors and single cells induces an increase in filopodial length, the duration of this effect differs. Global stimulation with the NO donor SIN-1, results in a long-term stimulation and induces a rapid and transient increase in filopodial length followed by a sustained plateau during which filopodia remain elongated over control values (Van Wagenen et al., 1999; Van Wagenen and Rehder, 2001). Local and short term stimulation with the NO donor NOC-7 causes a transient increase in filopodial length lasting approximately 20 minutes (Welshhans and Rehder, 2005; Welshhans and Rehder, 2007). Stimulation by a single cell, which continuously releases NO and results in long term stimulation, caused filopodial elongation and filopodia remained elongated in the presence of the stimulating cell (Tornieri and Rehder, 2007). These results suggest that the duration of the NO stimulation influences the time filopodia remain extended. Our finding that NO release from a single cell affects growth cone morphology of neighboring growth cones entering the “plume” of

NO strongly confirms a paracrine role of NO in regulating growth cone dynamics (Tornieri and Rehder, 2007). This concept of NO acting in a paracrine fashion was demonstrated during the development of the grasshopper embryo, where NO released from epithelial cells facing the basal lamina of the antenna regulates axonal pathfinding of antennal pioneer neurons (Seidel and Bicker, 2000; Bicker, 2007).

The fact that NO has a very short half-life, and the finding that NO release from a single cell affects filopodial length with the same intensity as stimulation with NO donors suggests that low concentrations of NO can have strong physiological effects on growth cone morphology. These strong changes in filopodial dynamics may be explained by amplification of spatial and temporal stimulation by NO through its signaling pathway, or that the system is very sensitive to begin with and is saturated easily. This effect of a low concentration of NO on filopodial length is also observed when NO is temporally and spatially applied on B5 growth cones for milliseconds with a picospitzer (Welshhans and Rehder, 2005). This strong effect on growth cone dynamics may be explained by the fact that binding of NO to the heme group of sGC increases its catalytic activity by a hundred fold (Bellamy et al., 2002; Russwurm and Koesling, 2004). It has been demonstrated that physiological NO concentrations in the brain are in the low nanomolar range (Hall and Garthwaite, 2006). NO sensing electrodes and fluorescent dyes such as DAF-2A have been used to detect NO release from tissues and cells within tissues (Friedemann et al., 1996; Bedioui et al., 1997; Kojima et al., 1998). Recently, detection of NO release from single neurons has been performed in the isolated CNS of *Lymnaea stagnalis* by using multiple film coated electrode sensors (Patel et al., 2006). Furthermore, picomolar concentrations of NO released from single epithelial cells and hippocampal neurons were detected by a cell-based indicator (Sato et al., 2006). Detection and quantification of NO released

from single B5 senders will be important to investigate in the future. Our study with the sender-receiver paradigm demonstrates that B5 neurons spontaneously produce NO and that NO has physiological effects on neighboring growth cones up to a distance of 100 μm away from the sender cell. This finding provides crucial physiological information to previous modeling studies of NO release, which have demonstrated a diffusion radius of approximately 200 μm from a point source of NO (Lancaster, 1994; Wood and Garthwaite, 1994; Lancaster, 1997). **Our finding provides the first demonstration that physiological concentrations of NO affect filopodial dynamics and strongly suggests a role of NO as a modulator of growth cone motility during neuronal pathfinding *in vivo*.**

Paracrine versus autocrine role of NO

The fact that B5 neurons express both nNOS and the NO target, soluble guanylyl cyclase (sGC) (Van Wagenen and Rehder, 2001), make them unique in that they produce NO and also can respond to it. Therefore, we can speculate that in addition to responding to NO release from neighboring cells during pathfinding B5 neurons may also intrinsically produce NO to regulate aspects of their own physiology. Indeed, inhibition of endogenous NOS in B5 neurons induces a slow-down in the rate at which neurites advance (Trimm and Rehder, 2004), suggesting that their own source of NO is necessary for a normal outgrowth rate, and down- or up-regulation of NO production may be an intrinsic mechanism to regulate neurite advance. In our study investigating the effect of NO release from a single cell on growth cone dynamics, inhibition of endogenous production of NO in B5 receivers with the specific NOS inhibitor 7-NI had no effect on filopodial dynamics (Tornieri and Rehder, 2007). This result suggests that endogenous NO may regulate neurite outgrowth by a mechanism independent from that regulating filopodial length.

Because nNOS is activated by calcium/calmodulin, one possible mechanism to regulate the amount of endogenous NO production could be through changes in the intracellular calcium concentration, which is modulated by neuronal activity. It will be of interest in the future to investigate if there is an interaction between endogenous and exogenous NO, with the rationale that the amount of spontaneously produced NO may modulate the response of the cell to exogenous NO. The fact that (1) NO signaling leads to an increase in $[Ca^{2+}]_i$ (Van Wagenen and Rehder, 1999), and (2) $[Ca^{2+}]_i$ regulates neurite outgrowth based on the calcium set point hypothesis (Kater and Mills, 1991), allows one to envision a scenario in which endogenously produced NO helps to ‘set’ the $[Ca^{2+}]_i$ which can be further modulated by exogenous NO to regulate growth cone behavior and neurite outgrowth. Modulation in the $[Ca^{2+}]_i$ has been shown to have striking effects on growth cone behavior, such as in the case of *Xenopus* spinal neurons, where changes in the amplitude of total $[Ca^{2+}]_i$ elevation were demonstrated to convert growth cone steering from attraction to repulsion (Zheng, 2000).

Interaction between Filopodial Length, Number and Neurite Outgrowth

Slow down and search behavior

As mentioned earlier, filopodia on growth cones are motile and sensing structures crucial for correct neuronal pathfinding. Although many studies have established the mechanisms by which filopodial dynamics govern growth cone behaviors, such as collapse and steering, the relationship between filopodial length and neurite outgrowth is still poorly understood. During their journey toward their targets and depending on their position in the environment, growth cone morphology and neurite outgrowth vary. Growth cones tend to have an elongated shape without any filopodia and advance at a fast rate when they grow along an already established

path laid down by other axons; whereas when growth cones are at decision points, filopodial length increases and the speed of neurite advance decreases (Tosney and Landmesser, 1985; Bovolenta and Mason, 1987; Godement et al., 1994; Kuhn et al., 1995; Trimm and Rehder, 2004). The latter has been referred to as a “slow down and search” behavior (Trimm and Rehder, 2004). The benefit of this behavior for a navigating growth cone would be to enhance its sensory ability: (i) by increasing its action radius through filopodial elongation, and (ii) by increasing the duration of its exploration of the environment.

This slow down and search behavior was also observed following inhibition of PI-3K. Interestingly, inhibition of either ROCK or Akt, two downstream targets of PI-3K, allowed us to separate the slow down and search behavior into a “search” and a “slow down” component (Tornieri et al., 2006). We can envision that during pathfinding *in vivo*, depending on the signaling pathway activated by guidance cues or the intensity of the stimulation, navigating growth cones may increase their action radius and continue to advance at the same speed (“search”) or, alternatively, increase their action radius and decrease or stop their advance (“slow down and search”). Because of technical issues, neurite outgrowth could not be investigated in our study using the sender-receiver paradigm, however, a previous publication from our laboratory demonstrated that NO induces a concomitant increase in filopodial length and a decrease in neurite outgrowth (Trimm and Rehder, 2004). The concept that the same signaling molecule may affect filopodial dynamics and neurite outgrowth is further supported by findings from multiple studies demonstrating that protein kinases affect both growth cone behavior and neurite outgrowth (Bixby, 1989; Bixby and Jhabvala, 1992; Oberstar et al., 1997; Cheng et al., 2000)

Calcium and growth cone dynamics

Calcium has been demonstrated to be a critical regulator of growth cone behavior, with an increase in $[Ca^{2+}]_i$ leading to filopodial elongation and a decrease in filopodial number (Kater and Mills, 1991; Rehder and Kater, 1992). However, filopodial elongation induced by inhibition of PI-3K is not mediated by an increase in $[Ca^{2+}]_i$, suggesting that the slow down and search behavior can be elicited by a calcium-independent mechanism as well (Tornieri et al., 2006). Furthermore these findings suggest that a calcium-dependent signaling event, like NO signaling, and a calcium-independent signaling event may converge on the same target to affect actin cytoskeleton dynamics and therefore elicit the same effect on filopodial dynamics and neurite outgrowth. Actin binding proteins (ABPs), such as cofilin and gelsolin, are good candidates for convergence targets, as they have been shown to be regulated by calcium, phosphatidylinositolphosphates and their phosphorylation state (Schafer et al., 1996; Meberg et al., 1998; Sun et al., 1999). The involvement of these ABPs in filopodial dynamics will be discussed in detail later in the discussion. However we cannot exclude the alternative possibility that the absence of measurable changes in $[Ca^{2+}]_i$ after PI-3K inhibition could be due to a limitation in the sensitivity of the calcium indicator dye. This explanation is in agreement with our finding that NO release from a single cell induces filopodial elongation without measurable changes in the $[Ca^{2+}]_i$, as opposed to elongation induced by NO donors. Assuming that the concentration of NO release in the sender-receiver paradigm is much smaller than the release from NO donors, the resulting increase in $[Ca^{2+}]_i$ may not to be detectable but sufficient to affect growth cone dynamics. Interestingly, we noticed that treatments that induce filopodial elongation without increasing the $[Ca^{2+}]_i$, such as PI-3K inhibition or NO release from a single cell, do not affect filopodial number. The same was observed in a previous study in *Helisoma* following

inhibition of tyrosine kinases and PKC (Cheng et al., 2000). These results suggest that: (i) filopodial length can be regulated independently of an elevation in $[Ca^{2+}]_i$ or (ii) filopodial length is more sensitive to small, possibly undetectable increases in $[Ca^{2+}]_i$ than filopodial number. The latter is supported by a study in *Helisoma*, in which a small, local increase in $[Ca^{2+}]_i$ by uncaging calcium resulted in filopodial elongation but had no effect on filopodial number (Cheng et al., 2002). This study suggests that a small increase in $[Ca^{2+}]_i$ affects filopodial length alone, and not filopodial number.

Actin Binding Proteins and Cytoskeletal Dynamics Underlying Changes in Growth Cone Filopodia

Filopodia are composed of bundles of F-actin and any changes in filopodial length will be mediated by changes in F-actin dynamics. Filopodial elongation can be explained by three mechanisms: (i) an increase in actin polymerization at the barbed end of F-actin, which is located at the tip of filopodia, (ii) a decrease in retrograde flow of F-actin, or (iii) a combination of both. Our finding that inhibition of actin polymerization with cytochalasin B blocked the filopodial elongation induced by PI-3K inhibition (Tornieri et al., 2006), confirms that filopodial length is mediated by actin dynamics, and more specifically suggests that inhibition of PI-3K increases actin polymerization at the barbed end. Our hypothesis that filopodial elongation is mediated by an increase in actin polymerization is further supported by a study in *Helisoma*, where NO donor-induced filopodial elongation was mediated by addition of actin monomers at the barbed end (K. Welshhans, personal communication). Because simultaneous inhibition of PI-3K and retrograde flow had an additive effect on filopodial elongation, we cannot exclude a role of PI-3K in regulating retrograde flow. However, our findings that inhibition of PI-3K does not affect

filopodial retraction parameters (retraction being thought to be driven by retrograde flow), and that a previous study in *Helisoma* demonstrated that a calcium-induced slow down in retrograde flow is dependent on changes in actin bundle dynamics in filopodia (Welnhofer et al., 1999), strongly exclude a mechanism involving mainly retrograde flow. The fact that NO-induced filopodial elongation is mediated by affecting the rate at which filopodia elongate and the time that individual filopodia spend elongating, but had no effect on retraction parameters (Van Wagenen et al., 1999), also supports our hypothesis that a slow-down of retrograde flow is not the main mechanism involved in filopodial elongation. To summarize, the above findings suggest that PI-3K inhibition and application of exogenous NO induce filopodial elongation by increasing actin polymerization at the barbed end. Actin polymerization is regulated by actin binding proteins (ABPs), which control branching, capping and polymerization. Spatial and temporal modification of the activity of these proteins will change the actin dynamics of the growth cone cytoskeleton, which is necessary for correct pathfinding.

There are multiple mechanisms by which PI-3K and NO might regulate F-actin extension. The actin binding protein, profilin, controls actin dynamics by binding and sequestering actin monomers, leading to the inhibition of actin assembly (Stossel, 1989). Phosphatidylinositol-phosphates, such as phosphatidylinositol-3,4-bisphosphate (PIP₂), have been demonstrated to prevent binding of profilin to actin monomers (Goldschmidt-Clermont et al., 1991; Lu et al., 1996). In addition, phosphorylation of profilin by PI-3K increases its affinity for actin monomers (Sathish et al., 2004). Therefore, PI-3K may regulate growth cone dynamics via profilin, with the rationale that inhibition of PI-3K increases F-actin polymerization by preventing phosphorylation of profilin and by maintaining a high amount of PIP₂. This would

then lead to an increase in the amount of free actin monomers available for addition at the plus end.

Another ABP, gelsolin, severs actin filaments and prevents actin polymerization onto F-actin by capping its barbed ends. Gelsolin is regulated by phosphatidylinositol-4,5-bisphosphate (PIP₂) which causes the dissociation of gelsolin from F-actin, and by calcium which promotes gelsolin binding to F-actin (Schafer et al., 1996; Sun et al., 1999). It may be that PI-3K inhibition induces actin polymerization by increasing the amount of PIP₂ available, and therefore uncapping gelsolin from barbed ends to promote actin polymerization (Fig. 5.1). Because NO induces filopodial elongation by increasing $[Ca^{2+}]_i$, and calcium activates gelsolin, NO may mediate its effect on actin polymerization through gelsolin activity. This hypothesis is supported by the finding that gelsolin promotes actin polymerization by first severing F-actin and then uncapping the newly generated actin filaments (Sun et al., 1999; Yin and Stull, 1999)

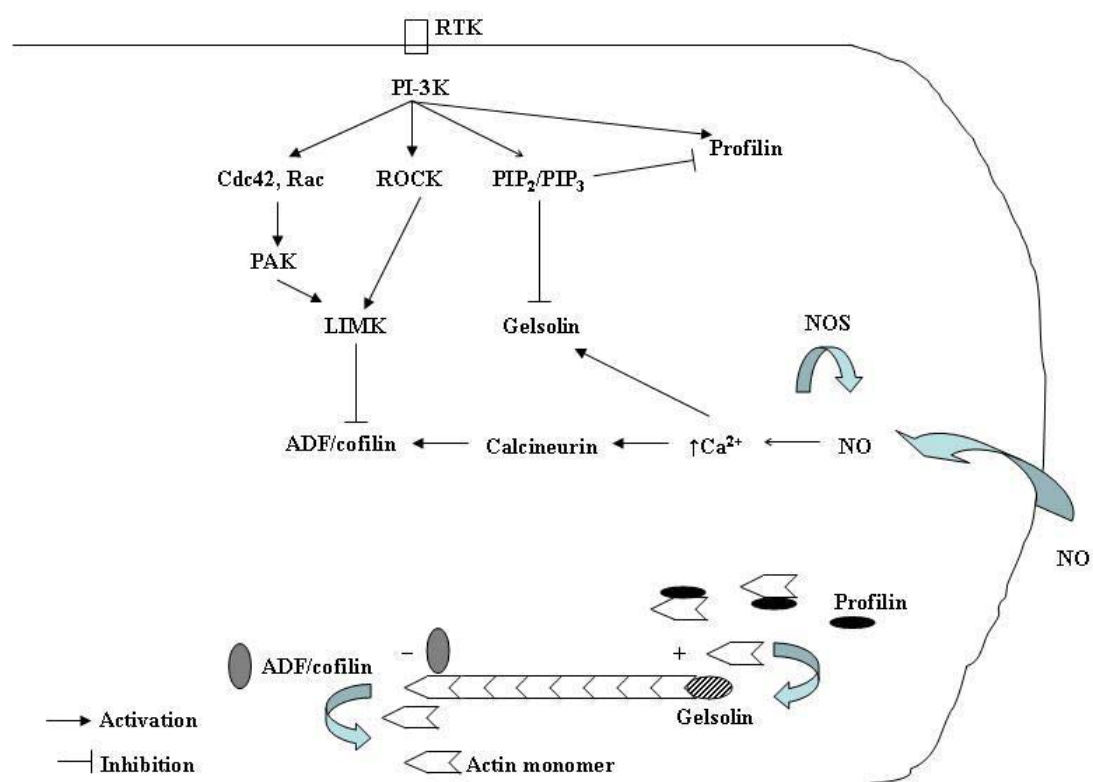
Finally, PI-3K and NO could also regulate actin polymerization via the actin depolymerizing factor ADF/cofilin, which increases the rate of dissociation of actin monomers from the pointed end of F-actin and severs F-actin (Bamburg, 1999; Chen et al., 2000; Sarmiere and Bamburg, 2004). The ability of ADF/cofilin to bind to F-actin is inhibited by phosphorylation on serine 3 by LIM kinase (LIMK) and by binding to phosphatidylinositol-phosphates (Yonezawa et al., 1991; Agnew et al., 1995; Arber et al., 1998; Sarmiere and Bamburg, 2004). Dephosphorylation of ADF/cofilin by phosphatases such as calcium/calmodulin-dependent phosphatase 2B (calcineurin) and slingshot activates ADF/cofilin (Meberg et al., 1998; Niwa et al., 2002; Sarmiere and Bamburg, 2004). PI-3K can inactivate ADF/cofilin via its lipid kinase activity by activating ROCK, which in turn activates LIMK (Maekawa et al., 1999). LIMK then phosphorylates and deactivates ADF/cofilin. Another

pathway to inactivate ADF/cofilin may be through its protein kinase activity by activation of Rac and Cdc42, which in turn activate the protein kinase (PAK), which phosphorylates LIMK (Manser et al., 1994; Edwards et al., 1999). Taken together, inhibition of PI-3K may induce actin polymerization by inhibition of the pathways mentioned above. This proposal is supported by a recent study on chick retinal neurons, in which BDNF induces filopodial elongation by activation of ADF/cofilin through inhibition of ROCK (Gehler et al., 2004). In addition to mediating filopodial elongation induced by inhibition of PI-3K, ADF/cofilin may regulate NO-induced filopodial elongation as well. Because NO induces an increase in $[Ca^{2+}]_i$ and dephosphorylation of ADF/cofilin can be caused by an increase in $[Ca^{2+}]_i$ and the calcium/calmodulin-dependent phosphatase, calcineurin (Meberg et al., 1998), we can envision that by increasing $[Ca^{2+}]_i$, NO activates calcineurin, which dephosphorylates and activates ADF/cofilin, providing more actin monomers available for polymerization at the barbed end of F-actin. **In summary, PI-3K and NO play an important role in regulating F-actin dynamics in growth cone filopodia during pathfinding. ABPs such as profilin, gelsolin and cofilin are the likely points of convergence of different signaling pathways and, therefore, modification of their activity will influence growth cone behavior (Fig. 5.1).**

Figure 5.1: Possible PI-3K and NO signaling pathways regulating filopodial length.

Activation of PI-3K by RTK leads to activation of Cdc42, Rac and ROCK, which in turn activates PAK and LIMK, respectively. Activation of PAK and LIMK, in turn, lead to the phosphorylation and subsequent inactivation of ADF/cofilin. PIP₂ and PIP₃, produced by PI-3K, inactivate capping proteins such as gelsolin and the actin monomer sequestering protein profilin. The activity of profilin can be enhanced via phosphorylation by PI-3K. An increase in [Ca²⁺]_i by endogenous and exogenous NO activates calcineurin, which in turn dephosphorylates and activates ADF/cofilin. [Ca²⁺]_i also activates gelsolin.

PI-3K: phosphatidylinositol-3 kinase; RTK: receptor tyrosine kinase; ROCK: RhoA activated protein kinase; PIP₂: phosphatidylinositol-bisphosphates; PIP₃: phosphatidylinositol-triphosphates; LIMK: LIM kinase; [Ca²⁺]_i: intracellular calcium concentration; ADF: actin depolymerizing factor.



Conclusions

Taken together, the studies presented in this dissertation provide new insights into the signaling mechanisms that control growth cone dynamics.

In Specific Aim 1 (Chapter 2), we demonstrated a role of PI-3K in regulating growth cone dynamics and motility. Inhibition of PI-3K induces both an increase in filopodial length and a decrease in the rate of neurite outgrowth. These effects were mediated by the lipid and protein kinase activities of PI-3K, resulting in the activation of Akt, ROCK and MEKK respectively. Filopodial elongation induced by inhibition of PI-3K is mediated by an increase in the time individual filopodia spend extending and by an increase in the rate at which filopodia extend. We also have demonstrated that filopodial elongation resulting from the inhibition of PI-3K is mediated by an increase in actin polymerization at the barbed end. Therefore, these results demonstrate that PI-3K and its downstream targets can regulate growth cone dynamics, which are known to be crucial for correct neuronal pathfinding *in vivo*.

In Specific Aim 2 (Chapter 3), we have shown that physiological concentrations of NO can affect growth cone dynamics. NO release from a single cell induces a rapid increase in filopodial length. This effect on filopodial dynamics is reversible, mediated by the canonical target of NO, sGC, and limited to the stimulated growth cone. Cellular NO can have a physiological effect on filopodial dynamics at a distance of up to 100 μm . Therefore, these results demonstrate that physiological release of NO can affect growth cone morphology.

The proper functioning of the nervous system depends on the formation of correct connectivity during development. Misguidance or failure to wire correctly can lead to clinical disorders often related to cognitive and mental illness, or to neuromuscular dysfunction, which occurs in spinal muscular atrophy. A combination of clinical and cell culture studies have started to describe the molecules and mechanisms underlying these diseases. *In vitro* studies often allow a deeper understanding into the molecular and cellular mechanisms regulating many processes of neuronal development, such as neuronal survival, differentiation, motility, pathfinding and synaptogenesis, because they simplify the complexities that occur when studying these events *in vivo*. Our findings on how NO and PI-3K affect filopodial dynamics provide novel insight into the cellular mechanisms controlling growth cone motility *in vitro*, which is known to be critical for correct pathfinding and establishment of appropriate connectivity. Studies focusing on single molecules provide critical information on signaling pathways, but *in vivo*, multiple molecules and their signals interact with each other to govern a specific growth cone behavior. Therefore in the future, to better understand growth cone behavior *in vivo*, studies will need to focus on how growth cones integrate different signals, and on mechanisms involved in modulating growth cone responses to specific guidance cues. Follow up studies to the *in vitro* assay used here will need to test the role of identified molecules and second messengers affecting growth cone dynamics *in vivo*. This will allow for an understanding of their role in the development of the nervous system.

In conclusion, these studies not only provide new information about signaling pathways and second messengers regulating growth cone dynamics, but also contribute to our understanding of the mechanistic basis of wiring defects associated with brain abnormalities. Most importantly, a better understanding of the cellular and molecular mechanisms regulating growth cone motility during the development of the nervous system may provide the foundation

for clinical approaches to enhance correct navigation and appropriate connectivity of neuronal circuitry to promote regeneration and repair following nerve injury and degeneration.

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