INTRACELLULAR MECHANISMS UNDERLYING GROWTH CONE COLLAPSE

by

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ABSTRACT

During the course of development, expression of attractive and inhibitory guidance cues play a pivotal role in the pathfinding decisions of a growing neuron. In addition, injury-induced recapitulation of their expression, particularly inhibitory cues, likely influences the course of axonal regeneration, thus providing a rationale for the intense focus in this area of research. Though significant progress has been made, it remains poorly described what signaling cascades, and in what combination, are involved during inhibitory-cue induced growth cone collapse. Therefore, to further understand why neurons are repelled or inhibited by certain cues, the aim of this thesis is to identify the underlying intracellular mechanisms regulating growth cone collapse induced by inhibitory cues. Using a novel anti-invasive compound called Motuporamine C (MotC), I have characterized in chapter 2 its effects as a regulator of neuronal outgrowth. I found that MotC was a robust stimulator of growth cone collapse leading to a cessation of neurite growth. This was partially mediated through Rho-ROCK signaling, a pathway involved in regulating actin dynamics. Based on this partial response, I hypothesized that other signal transduction pathways were involved. I addressed this in chapter 3 by identifying calcium-activated calpain, a protease well-characterized in playing a role in adhesion regulation, was also activated during MotC-induced growth cone collapse. Furthermore, I show that concurrent inhibition of both Rho-ROCK and calpain pathways are necessary for maximum attenuation of the MotC-mediated collapse response. Since these results were identified using an organic molecule not endogenously expressed in vertebrate organisms, I hypothesized in chapter 4 that similar pathways would be activated in response to a physiological in vivo guidance cue. Using the inhibitory cue
Semaphorin 5B (Sema5B), I found in addition to the activation of calpain, the phosphatase calcineurin was also involved in mediating Sema5B-induced growth cone collapse. Moreover, it is the combination of calpain- and calcineurin-mediated pathways that is required for evoking maximal growth cone collapse and that cross-talk between these two effector molecules occurs. These results are of particular interest since previously it was proposed by Gomez and Zheng (2006) that calpain and calcineurin signaling cascades were parallel pathways. Taken together, my findings show that different inhibitory cues activate multiple intracellular pathways that appear to impinge on different aspects of the intracellular machinery regulating motility. The combinatorial activation of these pathways is necessary for mediating maximal growth cone collapsing effects. Moreover, the elucidation of common signaling cascades between inhibitory cues to induce growth cone collapse may eventually provide novel targets for the development of new therapeutic strategies to promote functional recovery following neuronal injury.
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1. INTRODUCTION

A fundamental question in neuroscience is how the nervous system forms its elaborate architecture. Specifically, what are the precise morphological steps required for the development of the functional circuitry underlying the nervous system? Neurons must first migrate to specific locations, extend axons and dendrites towards their proper targets, and then form functional synapses. Following development, many of these complex processes are recapitulated during learning, memory, and neuronal regeneration. Due to the importance of these morphological steps during development, gaining a greater understanding of the underlying mechanisms governing these processes remains intensely studied.

The growth cone, a specialized structure at the motile tip of an extending neurite, was first described by Santiago Ramón y Cajal in 1890 as a “battering ram” that traverses through obstacles in the environment en route to its final connective target (Cajal, 1890). Since Ramón y Cajal’s landmark observations over a century ago, growth cone behaviour has been examined in detail. Stereotyped growth cone behaviours include pause, collapse, retract, and turning. These distinct actions reflect the growth cone’s ability to encounter and decode cues in the extracellular environment both temporally and spatially, and then modulate appropriate intracellular mechanisms in response to these cues (Henley et al., 2004; Mattson et al., 1988; McCobb et al., 1988; Wong et al., 2002). Though fundamental during development and following neuronal injury, the complex mechanisms associated with growth cone motility remain poorly resolved. Moreover, the studies that have examined the signaling cascades involved tend to focus on individual
pathways. Therefore, the aim of this thesis is to identify the underlying mechanisms, and their combinatorial activities, that govern growth cone motility. Specifically, I will address what signal transduction pathways are involved in inhibitory cue-induced growth cone collapse. In chapter 2, I will characterize a novel anti-invasive compound termed Motuporamine C (MotC) as a robust stimulator of growth cone collapse and provide evidence of a potential signal transduction pathway utilized by MotC. Based on the partial involvement of this putative pathway in MotC-mediated growth cone collapse, I will show in Chapter 3 the involvement of a second pathway that acts in a combinatorial fashion to mediate the collapsing effect of MotC. In Chapter 4, I will examine whether the putative pathways identified by MotC apply to a physiological in vivo guidance cue, Semaphorin 5B (Sema5B). By gaining a greater understanding of the intracellular pathways controlling outgrowth inhibition, the development of new therapeutic strategies to promote regeneration following neuronal injury may be expedited.

1.1 Cytoskeletal dynamics mediating growth cone motility

The growth cone is a dynamic structure at the tip of the neurite responsible for determining directional outgrowth. It does this by decoding informational cues from the extracellular environment and translating this into appropriate signal transduction pathways that ultimately impinge on the growth cone’s cytoskeleton to regulate motility (Bentley and O’Connor, 1994; Tanaka and Sabry, 1995). The cytoskeleton is composed of microtubules, actin microfilaments, and intermediate filaments. The growth cone can be divided into three regions; the peripheral, transient, and central domains (Fig. 1.1)
Figure 1.1. Chicken dorsal root ganglion growth cone stained for F-actin (red) and microtubules (green). (A) Normal motile growth cone differentiated regionally by peripheral (P-), transitional (T-), and central (C-) domains. Hollow arrowhead highlights filopodia and hollow arrow indicates lamellipodia. (B) Collapsed growth cone highlighting the dramatic loss of structure, in particular, in the P-domain.
The peripheral (P-) domain contains the bulk of filamentous actin (Dent and Gertler, 2003; Forscher and Smith, 1988). This actin is localized within finger-like projections termed filopodia and veil-like structures termed lamellipodia (Dent and Gertler, 2003; Meyer and Feldman, 2002). The central (C-) domain is rich in microtubules and contains organelles and vesicles (Gordon-Weeks, 2004). Lastly, the transitional (T-) domain serves as an interface between the P-domain and C-domain (Forscher and Smith, 1988; Gordon-Weeks, 2004). In the case of growth cone collapse, these regions become much less defined, with the greatest contraction occurring in the P-domain (Fig. 1.1). While it has been established that both microtubule and actin components play a major role in growth cone motility (Bentley and O'Connor, 1994; Smith, 1988), intermediate filaments are only found within neurites and therefore not considered to play a major role in outgrowth (Phillips et al., 1983; Shaw et al., 1985).

**Actin dynamics**

An actin filament (F-actin) consists of a double helical polymer composed of globular actin subunits (G-actin) (Dent and Gertler, 2003). They are polar in nature, with net growth and addition taking place at the plus (or barbed) end and disassembly occurring at the minus (or pointed) end (Pollard and Borisy, 2003). The barbed end is oriented towards the distal membrane, while the pointed end is directed inwards (Pollard and Borisy, 2003). While monomers can associate and dissociate from both the barbed and pointed ends in vitro, addition of G-actin on the barbed end and removal of
monomers on the pointed end is kinetically favoured (Pollard and Borisy, 2003).

Retrograde transport of F-actin towards the central domain of growth cones occurs in both filopodia and lamellipodia and is a myosin-driven process (Dent and Kalil, 2001; Diefenbach et al., 2002; Lin et al., 1996; Lin and Forscher, 1993), although the exact type(s) of myosin isoform(s) involved remains unclear.

The observation that actin is localized to the leading edge of the growth cone highlighted its importance in motility (Bray and Chapman, 1985; Forscher and Smith, 1988; Yamada et al., 1970; Yamada et al., 1971). In the P-domain of the growth cone, the bulk of filamentous (F-) actin forms two types of arrays. The first is a polarized bundled array of F-actin that composes the core of filopodia. Filopodia play an essential role in sampling the environment for guidance cue information and transduce these signals into directionality and outgrowth (Bentley and Toroian-Raymond, 1986; Bray and Chapman, 1985; Chien et al., 1993; O'Connor and Bentley, 1993). The second array that F-actin can adopt is a meshwork array that forms lamellipodia. These veil-like structures are thought to be important for growth cone protrusion and play a significant role in substrate adhesion during growth cone motility (Conklin et al., 2005; Jay, 2000). It has been shown that lamellipodial extension between filopodia encountering a positive cue leads to turning (Bentley and Toroian-Raymond, 1986; Kleitman and Johnson, 1989; O'Connor et al., 1990). Previously, it was proposed that actin served as a physical barrier to prevent microtubules from invading the P-domain of the growth cone (Forscher and Smith, 1988), although it has since been shown that microtubules can and do invade the periphery (Gordon-Weeks, 1991; Tanaka and Kirschner, 1995), indicating a much more active role for microtubules in motility.
**Microtubule dynamics**

Microtubules are an integral component of the cytoskeletal machinery important for motility. They are present along the neurite shaft as well as the growth cone (Fig. 1.1). Within the neurite, microtubules are bundled, but within the C-domain of the growth cone, they tend to be de-fasiculated (Tanaka and Sabry, 1995; Yamada et al., 1971). As with actin, microtubules have a polarized distribution in axons, with the fast-growing (plus) end directed towards the growth cone periphery, while the slow-growing (minus) end is oriented proximally towards the cell body (Dent et al., 1999; Dent and Kalil, 2001).

Microtubules are hollow cylindrical filaments composed of 13 protofilaments, comprised of $\alpha$ and $\beta$ tubulin subunits (Gordon-Weeks, 2004). Microtubule populations can be classified as either stable, characterized by acetylation of tubulin as a post-translational modification (Kozminski et al., 1993), or dynamic, characterized by tyrosination of tubulin post-translationally (Lai et al., 1994). Stable microtubules are typically localized along the neurite shaft and in the C-domain of the growth cone (Lim et al., 1989). Dynamic microtubules exist not only in the C-domain, but can also extend into the actin-rich P-domain, and at times the proximal region of the filopodium (Bush et al., 1996; Gordon-Weeks, 1991; Sabry et al., 1991; Zhou et al., 2002). Mitchison and Kirschner (1984a; 1984b) postulated that this dynamic instability afforded microtubules the ability to probe the actin network, allowing microtubules to be “captured” by the actin network in response to a morphogenetic signal and thus subsequently stabilized against catastrophe. Applied to the context of the motile growth cone, dynamic instability permits microtubules to investigate the actin network in the peripheral domain, and specifically
within the filopodia, for changes following an encounter with a guidance cue, upon which capture and stabilization can occur (Dent et al., 1999; Gordon-Wecks, 1991). This was first demonstrated using motile *Xenopus* spinal growth cones. By fluorescently visualizing microtubule direction within growth cones sharply turning at borders between laminin-permissive and collagen IV-non-permissive substrates, these experiments showed re-orientation of microtubules along the direction of turn (Tanaka and Kirschner, 1995).

**Adhesive Focal Contacts**

The cytoskeleton is intimately linked to the extracellular environment through the binding of cell adhesion molecules at sites termed focal contacts (Letourneau and Shattuck, 1989). The components which make up focal contacts include integrins, kinases, phosphatases, and the actin-associated proteins talin and vinculin (Gomez et al., 1996; Letourneau and Shattuck, 1989; Schmidt et al., 1995). Integrins serve as the migration-promoting receptors of the growth cone by acting as the primary link between the extracellular matrix and the actin cytoskeleton and have been shown to activate motility-related signaling cascades (Jay, 2000; Ridley et al., 2003).

One kinase family associated with adhesion regulation, Src tyrosine kinases, has been shown to regulate tyrosine phosphorylation at the tips of growth cone filopodia (Robles et al., 2005). By inhibiting Src kinase activity in the growth cone, Robles et al. (2005) demonstrated phospho-tyrosine signaling and downstream targets such as the focal contact marker vinculin were down-regulated, suggesting an uncoupling of the actin
cytoskeleton from adhesion receptors and thus resulting in diminished growth cone motility. Furthermore, previous studies have shown that guidance cue receptors such as Plexin-A2 and Plexin-B1 can regulate Src kinase activity to regulate motility (Basile et al., 2005; Sasaki et al., 2002). These results highlight the importance of signaling pathways governing focal contacts, and presumably the tractional force required, for neurite outgrowth. It is also important to note differences in adhesion requirements for non-neuronal versus neuronal cells. At the leading edge of non-neuronal cells, adhesion is necessary to provide the tractional forces necessary for forward movement, whereas at the trailing edge, de-adhesion is important to allow the cell’s rear to retract towards the direction of movement (Ridley et al., 2003). It is thus the balance between these two processes which promotes forward migration. In neurons, no trailing edge is present and thus retraction in this context is not observed (Dent and Gertler, 2003; Kalil and Dent, 2005). One exception may be the exploratory nature of filopodia as they sample the environment, although it remains unknown what the dynamic relationship between adhesion and de-adhesion is in this case.

**The molecular clutch model for directed growth cone motility**

To account for the interdependency of the cytoskeletal and adhesive elements governing the motile growth cone, the molecular clutch model was proposed to describe the force-coupling required for productive forward movement (Mitchison and Kirschner, 1988). Based on this model, an extending lamellipodium that encounters an extracellular ligand that binds a cell surface receptor on that lamellipodium will couple this complex to
the actin cytoskeleton, and thus engaging the “clutch” (Lin et al., 1996; Lin and Forscher, 1995; Sheetz et al., 1998). In turn, actomyosin contraction of actin filaments will not be able to pull the actin network back towards the proximal end of the growth cone. As a result, lamellipodial protrusion occurs by net actin assembly at the distal barbed end of the actin filament that is fixed with respect to the substrate (Mitchison and Kirschner, 1988). Providing protrusion is occurring as a result of an encounter with a chemoattractant cue in the environment, engorgement of the growth cone in this protrusive direction occurs (Goldberg and Burmeister, 1986). This involves microtubule capture in this protrusive region, and subsequently the transport of organelles and vesicles (Dent et al., 1999; Gordon-Weeks, 1991). Following engorgement, consolidation occurs as the proximal region of the growth cone assumes a cylindrical shape and organelle transport becomes bidirectional (Fig. 1.2). In addition, when this “clutch” is disengaged, retrograde flow resumes to direct proximal movement of F-actin (Forscher and Smith, 1988; Mitchison and Kirschner, 1988). When the rate of retrograde flow is faster than actin assembly, retraction of the lamellipodium occurs. The balance between actin assembly, the coupling of the growth cone cytoskeleton with cell adhesion molecules, and retrograde flow thus serves as a fundamental mechanism of motility.

**Cytoskeletal dynamics implicated in growth cone collapse**

Growth cone collapse represents an extreme motile event in which the cytoskeletal machinery contributing to motility appears to break down. Though not well defined, it appears collapse involves the retraction and/or depolymerization of both the
**Figure 1.2.** Schematic of the clutch model for directed neurite outgrowth. Adapted from Dent and Gertler (2003), Jay (2000), and Mitchison and Kirschner (1988). (A) Filopodial protrusion towards a chemoattractive cue in the context of the clutch hypothesis. Protrusion is dependent on the assembly of actin at the filopodial tip and a clutch mechanism to prevent actomyosin contraction on F-actin. (B,C,D) Directed neurite outgrowth towards a chemoattractive cue as defined by the stages of (B) protrusion, (C) engorgement, and (D) consolidation.
A

Filopodial protrusion

G-actin
Retrograde flow
Mycosin
Clutch

B

Neurite protrusion

C

D

Vesicle
Microtubule
Bundled F-actin
F-actin meshwork
Chemoattractive cue
Region of interest

12
actin and microtubule network from the growth cone periphery (Pollard and Borisy, 2003; Gordon-Weeks, 1991). This in turn results in filopodial and lamellipodial retraction. Presumably, this event also requires detachment from the extracellular surface to facilitate retraction, ultimately producing a collapsed phenotype characterized by a “club-like” appearance at the neurite tip (Fig. 1.1B).

1.2 Molecular mechanisms mediating growth cone motility

While the mechanisms underlying cytoskeletal rearrangement are relatively well defined, the identification of the signal transduction pathways regulating the protrusive and retractive properties of the cytoskeleton remains poorly described, and at times, contradictory. Previous studies from the Poo lab have identified cAMP as an important second messenger regulating the turning behaviour of growth cones (Henley and Poo, 2004; Song et al., 1998; Song et al., 1997). Ca\(^{2+}\) has also been shown to act as a key second messenger in modulating growth cone motility. In this case, the downstream effectors of Ca\(^{2+}\) are only beginning to emerge, with some of the identified targets being the phosphatase calcineurin (Lautermilch and Spitzer, 2000; Wen et al., 2004), the kinases Ca\(^{2+}/CaM\)-dependent protein kinase II (Wen et al., 2004) and protein kinase C (Jin et al., 2005; Sivasankaran et al., 2004), and the protease calpain (Robles et al., 2003). Furthermore, examination of the Rho family of small GTPases, the most widely studied of which are Rho, Rac, and Cell division cycle 42 (Cdc42), has proven their importance in regulation of motility, albeit at times contradictory (Jin and Strittmatter, 1997; Liu and Strittmatter, 2001; Luo, 2000). With Rho GTPases as the exception, identification of
these downstream effectors has been achieved primarily through direct manipulation of Ca\(^{2+}\) or cAMP levels within the growth cone (Lautermilch and Spitzer, 2000; Robles et al., 2003; Wen et al., 2004). Therefore, it remains unclear whether these signaling cascades are also regulated by extracellular cues involved in regulating growth cone motility. In addition, even less understood are what intracellular pathways modulate microtubule re-organization in the motile growth cone.

**Cyclic nucleotides**

Cyclic nucleotides have been shown to play important roles as second messengers. In the case of cAMP, it is converted from ATP via adenylyl cyclase, while cGMP is converted from GTP via guanylyl cyclase. Important studies from the Poo laboratory have identified the importance of cyclic nucleotides in regulating growth cone motility. Using a growth cone turning assay, Song et al. (1997) showed that the normally chemoattractive brain-derived neurotrophic factor (BDNF) could be switched to a chemorepellant by reducing intracellular cAMP levels. Follow-up studies demonstrated Sema3A-mediated growth cone repulsion could be switched to attraction by raising intracellular cGMP levels (Song et al., 1998). Based on these observations, it was proposed that the ratio of specific nucleotides could modulate the turning response of the motile growth cone (Song et al., 1998). To date, cues have been divided into two classes based on responsiveness to specific cyclic nucleotides. Cues sensitive to cAMP levels include nerve growth factor (NGF), BDNF, netrin, and myelin-associated glycoprotein (MAG), while cues sensitive to cGMP include sema3A, neurotrophin-3, and the
chemokine SDF-1 (Song et al., 1998; Song et al., 1997; Song and Poo, 1999; Xiang et al., 2002). Furthermore, evidence indicates that these increases in cyclic nucleotide levels result in a positive feedback loop (Bolsover, 2005; Ming et al., 2001). For instance, increases in [Ca$^{2+}$]i result in a raised cAMP:cGMP ratio, which in turn increases the open probability of cAMP-sensitive Ca$^{2+}$ channels/stores (Ming et al., 2001). Thus, the interrelationship between cyclic nucleotides and Ca$^{2+}$ further underscores the complexity of the molecular mechanisms regulating growth cone motility.

**Calcium**

Ca$^{2+}$ is another second messenger that plays a central role in transducing extracellular guidance information to outgrowth and directional motility. Regulation of the intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]i) in the growth cone is precisely maintained by Ca$^{2+}$ influx through plasma membrane Ca$^{2+}$ channels, Ca$^{2+}$ release from internal stores, and Ca$^{2+}$ extrusion through plasma membrane Ca$^{2+}$-ATPases and plasmalemmal Na$^+$-Ca$^{2+}$ exchangers (Bolsover, 2005; Henley and Poo, 2004; Henley et al., 2004; Hong et al., 2000). Based on this regulation, the Ca$^{2+}$ set-point hypothesis has been proposed, which states that an optimal range of [Ca$^{2+}$]i is required for normal growth cone motility and neurite outgrowth, while Ca$^{2+}$ fluctuations above or below this optimal concentration results in outgrowth inhibition (Kater and Mills, 1991). This hypothesis has been substantiated in numerous neuronal cell types (al-Mohanna et al., 1992; Gu et al., 1994; Henley and Poo, 2004; Lankford and Letourneau, 1991). Furthermore, the spatial presentation of an extracellular stimulus the growth cone...
encounters can dictate its motile behaviour. For example, in the case of extracellular bath application of dopamine or serotonin, a global high amplitude rise in [Ca\(^{2+}\)]\(_i\) was observed, leading to growth cone collapse and inhibition of outgrowth (Mattson et al., 1988; McCobb et al., 1988). On the contrary, when an extracellular gradient of glutamate or acetylcholine was presented, a moderate rise in [Ca\(^{2+}\)]\(_i\) was found, inducing an attractive growth cone steering response (Zheng et al., 1994; Zheng et al., 1996). These experiments not only support the Ca\(^{2+}\) set-point hypothesis, but also substantiate the idea that global changes in [Ca\(^{2+}\)]\(_i\) regulate neurite outgrowth, while asymmetric local changes in [Ca\(^{2+}\)]\(_i\) influence growth cone steering. Therefore, based on how an extracellular cue is spatially presented to a growth cone, one can begin to address questions specifically concerning outgrowth versus steering.

**Calcineurin, protein kinases, and calpain**

Recently, efforts have been made to identify the downstream effector molecules regulated by Ca\(^{2+}\) signaling. The most extensively studied Ca\(^{2+}\)-binding protein is calmodulin (CaM) (Bolsover, 2005). Upon binding, Ca\(^{2+}\)/CaM interacts with several targets, including Ca\(^{2+}\)/CaM-dependent protein kinases (CaMKs). Studies on the role of CaMKII, a kinase localized to the actin cytoskeleton and shown to regulate neurite extension, indicates it plays a central role in Ca\(^{2+}\)-dependent chemoattraction in *Xenopus* growth cones (Wen et al., 2004; Zheng et al., 1994). By modestly elevating local [Ca\(^{2+}\)]\(_i\), via photolysis of caged Ca\(^{2+}\), it was shown that CaMKII specifically promotes attractive growth cone turning and that this response is mediated via regulation of the Rho family
of GTPases (Jin et al., 2005; Wen et al., 2004). Another kinase, protein kinase C (PKC), has also been implicated in growth cone motility. It has been shown to be activated following increased [Ca$^{2+}$], stimulated by the chemoattractant, BDNF (Jin et al., 2005). Subsequently, PKC was found to inhibit Rho, while promoting Rac and cdc42 activity (Jin et al., 2005). Furthermore, pathways responsible for the repulsive behaviour in motility arising from low- and high-amplitude [Ca$^{2+}$]$_i$ increases have also begun to be teased out (Lautermilch and Spitzer, 2000; Robles et al., 2003; Wen et al., 2004). By inducing a low-amplitude rise in [Ca$^{2+}$]$_i$ via photolysis of caged Ca$^{2+}$ in Xenopus growth cones, Wen et al. (2004) demonstrated the Ca$^{2+}$/CaM-dependent phosphatase calcineurin is required for repulsive turning. Examination of potential substrates of calcineurin under these conditions led to the identification that both protein phosphatase 1 (PP1) and the growth and plasticity associated protein 43 (GAP-43) may be involved (Lautermilch and Spitzer, 2000; Wen et al., 2004). In the case of induction of large Ca$^{2+}$ transients, the Ca$^{2+}$-sensitive protease calpain has been shown to be activated and that calpain is necessary for growth cone repulsion in this case (Robles et al., 2003). Taken together, this has led to a recent revision of the Ca$^{2+}$-set point hypothesis; repulsion/inhibition via low-amplitude rises in [Ca$^{2+}$]$_i$ requires calcineurin activity, attraction/outgrowth via mid-amplitude rises in [Ca$^{2+}$]$_i$ require CaMKII or PKC, and repulsion/inhibition via high-amplitude rises in [Ca$^{2+}$]$_i$ require calpain (Gomez and Zheng, 2006) (Fig. 1.3). However, since most of these studies directly modulated Ca$^{2+}$ levels within the growth cone, it remains unknown whether this model holds true for most extracellular cues that regulate motility. Specifically, it is not known whether inhibitory cues that induce growth cone collapse through changes in [Ca$^{2+}$]$_i$ do in fact activate calcineurin- or calpain-mediated
**Figure 1.3.** Schematic highlighting signal transduction cascades regulated by different amplitude fluxes in \([\text{Ca}^{2+}]_i\). Low-, mid-, and high-amplitude increases in \([\text{Ca}^{2+}]_i\) are represented by small, medium, and large \([\text{Ca}^{2+}]_i\) schematic balloons, respectively. Varying \(\text{Ca}^{2+}\) signals are proposed to regulate distinct downstream pathways. Red schematic balloons indicate proteins typically associated with induction of growth cone collapse, while blue schematic balloons indicate proteins usually associated with promoting outgrowth. Adapted from Gomez and Zheng (2006).
Adapted from Gomez and Zheng (2006)
pathways. Moreover, since previous studies have shown that calcineurin is a proteolytic target of calpain (Shioda et al., 2006), it is unclear whether these pathways are mutually exclusive of one another during growth cone collapse.

**Rho GTPases and their downstream targets**

The small GTPases of the Rho family represent a major class of downstream second messenger effectors in growth cone motility. Critical regulators of the actin cytoskeleton, the most widely studied have been Rho, Rac, and Cdc42. These GTPases exist either in an inactive, GDP-bound state, or an active, GTP-bound state. Three protein classes regulate the nucleotide-binding state of this family: guanine nucleotide exchange factors (GEFs), which facilitate the exchange of GDP for GTP, GTPase activating proteins (GAPs), which increase the GTPase activity of this subfamily and thereby inactivate them, and guanine dissociation inhibitors (GDIs), which regulate both the GDP/GTP cycle and the membrane association/dissociation cycle (Luo, 2002). In neurons, Rho activation has been shown to mediate neurite retraction and growth cone collapse (Jalink et al., 1994; Katoh et al., 1998; Wahl et al., 2000). It is of interest to note that in non-neuronal cells, Rho activation differs slightly in that it results in stress fiber and adhesion complex formation; processes necessary for generating contractile forces to pull the cell forward during migration (Ridley, 2001). In the case of Rac and Cdc42 activation, these GTPases have, for the most part, been shown to promote neurite outgrowth, with Cdc42 thought to be involved in filopodial protrusion and Rac mediating lamellipodial formation (Brown et al., 2000; Kozma et al., 1997; Lamoureux et al., 1997;
Figure 1.4. Signaling cascades implicated in Rho GTPase activity in growth cone motility. Red schematic balloons indicate proteins typically associated with promoting growth cone collapse, blue schematic balloons show proteins usually associated with promoting outgrowth, while green schematic balloons have been shown to play roles in both outgrowth and inhibitory responses. It should be noted these signaling cascades do not represent all potential downstream targets of Rho GTPases associated with growth cone motility. Abbreviations: MRLC, myosin regulatory light chain; MRLC-Ph, myosin regulatory light chain phosphatase.
Luo et al., 1994). However, exceptions to the commonly ascribed functions of these three Rho GTPases have been identified, such as Semaphorin 3A signaling to induce outgrowth inhibition as described below (Jin and Strittmatter, 1997).

As a growth cone encounters extracellular guidance cues, a series of signaling cascades are triggered by the ligand-receptor complex. In many cases, these complexes initiate signal transduction pathways that eventually target Rho GTPases, which in turn regulate cytoskeletal dynamics (Fig. 1.4) (Gallo and Letourneau, 2003; Giniger, 2002; Luo, 2000). In the case of Rac and Cdc42, p21-activated kinase (PAK) is a common effector, and NGF-induced neurite outgrowth in PC12 cells is inhibited by dominant-negative forms of either GTPase (Daniels et al., 1998). Furthermore, PAK signaling has been shown to activate LIM-domain-containing protein kinase (LIM-kinase), which in turn phosphorylates and inhibits the actin depolymerizing factor, coflin, leading to protrusion (Arber et al., 1998; Yang et al., 1998). In the case of Rho, the best-studied effector is the serine/threonine Rho-kinase (ROCK). Rho-mediated ROCK activation has been shown in numerous neuronal cell types to promote growth cone collapse and neurite retraction (Bito et al., 2000; Hirose et al., 1998; Loudon et al., 2006; Niederost et al., 2002). Effectors downstream of ROCK include the regulatory light chain of myosin II (MRLC) and the regulatory subunit of myosin light chain phosphatase (MRLC-Ph), both of which in turn lead to increased phosphorylation of myosin light chain and ultimately myosin II-mediated retraction (Gallo et al., 2002; Loudon et al., 2006; Mulder et al., 2004; Schmidt et al., 2002). Adding to the complexity of these signal transduction pathways, ROCK can also activate LIM-kinase (Maekawa et al., 1999; Ohashi et al., 2000). As LIM-kinase represents a common target to both PAK and ROCK, it remains unresolved
how the seemingly disparate effects of Rac/cdc42 and Rho are mediated through convergent effector molecules. It should also be noted that in addition to ROCK, the formin-related protein, mammalian diaphanous (mDia), has been implicated in Rho-mediated signaling to regulate both actin assembly and microtubule stabilization and thus serves as another potential target involved in growth cone motility (Eisenmann et al., 2007; Watanabe et al., 1999; Watanabe et al., 1997).

**Glycogen synthase kinase-3β**

While significant progress has been made in identifying the molecular mechanisms regulating the actin cytoskeleton, few studies have examined the intracellular cascades governing microtubule dynamics during growth cone collapse. Most studies to date have teased out pathways implicated in microtubule re-organization after growth cone exposure to the repellent cue, lysophosphatidic acid (LPA). Upon binding to an endothelial differentiating gene (EDG) receptor family member, it has been demonstrated that in addition to inducing actin filament contraction via Rho-ROCK signaling, microtubule re-organization occurs through activation of the serine/threonine kinase, glycogen synthase kinase-3β (GSK-3β) (Sayas et al., 2002a; Sayas et al., 2002b). Subsequently, GSK-3β activates the microtubule binding protein, Tau, leading to microtubule instability and neurite retraction (Sayas et al., 2002a; Sayas et al., 1999). Furthermore, a recent study by Zhou et al. (2004) has shown that NGF-induced axon growth is regulated via inactivation of GSK-3β. This inactivation in turn led to an accumulation of the microtubule binding protein, adenomatous polyposis coli (APC), at
the growing plus ends of microtubules, suggesting APC plays a role in microtubule stabilization (Zhou et al., 2004). Future studies will be required to examine whether and how microtubule organization is modulated by other guidance cues.

1.3 Extracellular cues regulating growth cone motility

The activation of the intracellular mechanisms regulating growth cone motility is initiated by the extracellular cues the growth cone encounters. As an axon navigates through the extracellular environment, either during development or following neural injury in the peripheral nervous system, the motile growth cone must integrate guidance information from extracellular cues and translate this information into an appropriate physiological response. Cues can be classified into 3 categories: positive guidance cues (e.g. - netrins), negative guidance cues (e.g. - semaphorins), and myelin-associated inhibitors (e.g. – MAG). The growth cone response to these cues depends on the spatial context in which they are presented. In the case where a gradient is applied, the growth cone will turn towards attractive cues and away from negative cues, whereas if the cue is presented globally to the growth cone, outgrowth will be promoted with a positive cue and collapse/retraction with a negative cue (Gomez and Zheng, 2006; Henley and Poo, 2004; McFarlane, 2000). While the behavioural outcome of the motile tip reflects the spatial context of the signal, the downstream signaling cascades initiated are likely the same (Gomez and Zheng, 2006; Henley and Poo, 2004). However, how these pathways are regulated by specific cues to induce particular motile responses remains largely undefined. Previous studies have tended to focus on the involvement of individual
pathways modulated in response to a specific guidance cue. This most likely does not reflect the underlying mechanisms governing motility since it can be assumed that more than one pathway is activated during a given motile response. It is therefore of interest to elucidate which molecular mechanisms are regulated by extracellular cues that direct growth cone motility, and specifically collapse, and to examine whether combinatorial activation of multiple pathways is required.

**Semaphorins – A prototypical family of repellent guidance cues**

One of the most widely-studied guidance cue families, the semaphorins are secreted and membrane-associated glycoproteins that can be grouped into eight classes based on structure and amino acid sequence similarity. Class 1 and 2 are found in invertebrates, classes 3-7 are in vertebrates, and class 8 is encoded by viruses (Raper, 2000). All semaphorins contain a stereotypical ~500 amino acid Semaphorin domain (Kolodkin, 1998). Classes 2, 3, 4, and 7 contain a single immunoglobulin-like domain near the C-terminus, whereas class 5 semaphorins have a domain containing seven thrombospondin type 1 and type 1-like repeats (Adams et al., 1996; Raper, 2000). Furthermore, semaphorin 1, 4, 5, 6, and 7 are membrane-associated, while class 2, 3, and 8 are secreted (Raper, 2000). Though typically thought of as playing an inhibitory role in axonal guidance, it is now clear these molecules can be bifunctional, mediating repulsion and attraction in different neurons.

Plexins are the predominant family of semaphorin receptors and are categorized as isoforms A through D. It is thought plexins are autoinhibitory by nature, and that
semaphorin binding permits plexin activation (Takahashi and Strittmatter, 2001). Interestingly, semaphorin class 3 requires the transmembrane protein neuropilin as a co-receptor to signal through Plexin A (Antipenko et al., 2003). To date, the signal transduction pathways modulating this receptor-ligand interaction in growth cone motility remain poorly described. In the case of the inhibitory cue semaphorin 4D (Sema4D), upon binding Plexin B1, activated Rac is recruited to this receptor complex through a GTPase-binding domain in Plexin (Vikis et al., 2002; Vikis et al., 2000). Since active Rac normally promotes actin polymerization through Pak, it is thought that in this case Rac is sequestered, thus down-regulating Pak activity. Previous studies examining the prototypical inhibitory semaphorin 3A (Sema3A) indicates that upon binding to Plexin A1 and its co-receptor neuropilin-1, Rac co-localizes with this receptor complex (Fournier et al., 2000). In addition, a dominant-negative form of Rac inhibits Sema3A-induced growth cone collapse and outgrowth inhibition (Jin and Strittmatter, 1997). Examination of downstream targets of this signaling pathway have shown that Sema3A inhibits cofilin activity via LIM-kinase activation to induce growth cone collapse (Aizawa et al., 2001). Although the involvement of Rac in Sema3A signaling implicates this GTPase as a repressor of outgrowth and motility, it appears to be more the exception rather than the rule. Moreover, relatively few studies have examined Ca$^{2+}$ signaling in response to semaphorins. This is likely due to the fact that the prototypical Sema3A has been shown to mediate its repulsive effects in a Ca$^{2+}$-independent manner (Shim et al., 2005; Wen et al., 2004). Therefore, further studies are required to identify whether other semaphorins do in fact mediate their motile effects on growth cones through Ca$^{2+}$. 
Netrins – A prototypical family of attractive guidance cues

Netrins are laminin-like molecules that bind the receptors Deleted in Colon Cancer (DCC) and UNC5 to mediate distinct motile behaviours. When netrin binds to DCC, growth cone attraction occurs (Keino-Masu et al., 1996; Nikolopoulos and Giancotti, 2005), whereas binding to UNC5, either as homodimers or heterodimers with DCC, elicits repulsive effects (Hong et al., 1999; Nikolopoulos and Giancotti, 2005). In the case of growth cone attraction, a mid-amplitude increase in \([\text{Ca}^{2+}]_i\) has been shown to result from an influx of \(\text{Ca}^{2+}\) through voltage-dependent \(\text{Ca}^{2+}\) channels (VDCCs) and transient receptor potential (TRP) channels, as well as \(\text{Ca}^{2+}\) release from internal stores (Hong et al., 2000; Wang and Poo, 2005). Examination of the downstream effectors of netrin signaling identified an increase in Rac activity (Jin et al., 2005). To confirm that this Rac activity was indeed dependent on \(\text{Ca}^{2+}\), chelation of intracellular \(\text{Ca}^{2+}\) via BAPTA-AM abolished this effect (Jin et al., 2005). Whether the regulation of \([\text{Ca}^{2+}]_i\) and the downstream effects on Rho GTPases is required to mediate the motile effects of other extracellular cues remains undefined.

Myelin-associated glycoprotein – A myelin-associated inhibitor

MAG is an extensively studied, myelin-associated inhibitory protein belonging to the immunoglobulin superfamily. Normally found in both peripheral and CNS myelin, it is involved in the formation and maintenance of myelin sheaths (Fruttiger et al., 1995; Marcus et al., 2002). However, following injury to the CNS, the inhibitory nature of
MAG contributes to the non-permissive environment hindering axonal regeneration (Sandvig et al., 2004). MAG binds with high affinity to the Nogo receptor (NgR), but requires p75NTR as a co-receptor to stimulate inhibition of outgrowth and growth cone collapse (Wong et al., 2002). More recently, additional co-receptors of MAG signaling have been identified (Mi et al., 2004; Shao et al., 2005).

Examination of downstream second messengers of MAG signaling has revealed Ca\(^{2+}\) and cAMP to play central roles in MAG-mediated inhibition. It has been shown that MAG induces a low-amplitude rise in [Ca\(^{2+}\)], via Ca\(^{2+}\) release from internal stores (Hong et al., 2000). Furthermore, Song et al. (1998) demonstrated that MAG-mediated growth cone repulsion can be converted to attraction by elevating cAMP levels in *Xenopus* spinal neurons. Henley et al. (2004) sought to determine the underlying relationship between Ca\(^{2+}\)- and cAMP-dependent signaling following MAG addition. In this study, it was found that upon increased cAMP signaling, which converts MAG-mediated repulsion to attraction, basal levels of [Ca\(^{2+}\)] increased to a mid-amplitude response (Henley et al., 2004), providing further support for the Ca\(^{2+}\) set-point hypothesis. Finally, it has also been demonstrated that upon MAG/NgR/p75NTR association, Rho is activated via p75NTR (Vinson et al., 2001; Wong et al., 2002; Yamashita et al., 1999), while Rac and Cdc42 are concomitantly inactivated (Niederost et al., 2002). Therefore, similar to netrin-1, it appears that a common resulting mechanism of guidance cue stimulation is an amplitude rise in [Ca\(^{2+}\)], followed by the downstream activation of intracellular pathways involved in regulating cytoskeletal dynamics. Still, it remains unknown how broad this commonality is and specifically whether other negative cues induce similar signal transduction cascades to mediate outgrowth inhibition.
Motuporamine C – Utilization of an anti-invasive compound to study growth cone collapse

Motuporamines are a novel family of alkaloids isolated from the marine sponge, *Xestospongia exigua* (Williams et al., 1998). By utilizing an anti-invasive screen, Roskelley et al. (2001) found that Motuporamine C (MotC) inhibits the migration of a number of cancer cell lines. Furthermore, McHardy et al. (2004) showed MotC increases stress fiber formation and that this is due to an upregulation of Rho activity. However, little is known about the mechanisms of MotC function including what target MotC directly modulates and whether the target is intracellular or at the cell surface. Nevertheless, based on the results in non-neuronal cultures, and given the inhibitory role Rho plays in neuronal growth cone motility, MotC may serve as a useful research tool in elucidating the intracellular mechanisms underlying outgrowth inhibition in neurons.

1.4 Summary

Recent progress has begun to identify the signal transduction cascades involved in regulating outgrowth inhibition. However, how these pathways are modulated by extracellular cues remains unclear. Presently, it is unknown whether these pathways may crosstalk and/or act in concert with one another to mediate the motile response stimulated by a specific cue. This information would be of significant interest as it will identify potential key targets to develop more potent therapeutic strategies to stimulate neurite regeneration. Therefore, the aim of this thesis is to examine the signalling cascades that
underlie growth cone collapse and whether multiple pathways in combination are required. In chapter 2, I characterize the anti-invasive compound MotC as a robust collapsing cue of growth cones and provide evidence that MotC signals, at least partially, through the Rho-ROCK pathway. Based on my experiments in which inhibition of the Rho-ROCK pathway only partially attenuates the growth cone collapsing effects of MotC, I hypothesize in chapter 3 that other signaling cascades act in concert with Rho-ROCK. Indeed, I identify Ca\textsuperscript{2+}-mediated calpain activation as a second pathway regulated by MotC during collapse. Since MotC is an organic molecule not found in vertebrate organisms, I next examine whether similar intracellular pathways are modulated by a physiological \textit{in vivo} guidance cue. Using the inhibitory cue Sema5B in my growth cone collapse assay, combined with the robust Ca\textsuperscript{2+} response stimulated by MotC during outgrowth inhibition, I hypothesize in chapter 4 that Ca\textsuperscript{2+} and its downstream targets are involved in Sema5B-mediated collapse. I find that Ca\textsuperscript{2+} and the downstream targets, calpain and calcineurin, are involved. Interestingly, I also find in this case that the combined activation of calpain and calcineurin is necessary to promote complete collapse due to Sema5B signalling. Taken together, these studies show that inhibitory cues stimulate a complex network of downstream signal transduction pathways to induce growth cone collapse. I also show that it is indeed the activation of these pathways in particular combinations that contributes to the complete collapsing effects of particular cues. Based on these contributions, I propose future studies to begin examining the combined activation of multiple pathways targeting distinct cytoskeletal aspects of the growth cone necessary for motility.
**1.5 Research objectives**

In this thesis, I describe three research objectives.

1) Based on the anti-invasive property of MotC on non-neuronal cells, I will characterize the effects of MotC on growth cone motility and neurite outgrowth so that it may serve as a research tool to identify intracellular pathways regulating growth cone motility.

2) Utilizing MotC as a robust inhibitor of growth cone motility, I will identify signalling cascades regulated during growth cone collapse.

3) Since MotC is an organic molecule not endogenously present in vertebrates, I will examine whether the downstream targets identified in the second objective are regulated under more physiologically relevant conditions using the *in vivo* guidance cue, Sema5B.
1.6 References


2. THE ANTI-INVASIVE COMPOUND MOTUPORAMINE C IS A ROBUST STIMULATOR OF NEURONAL GROWTH CONE COLLAPSE

2.1 Introduction

Cell motility is a fundamental process for all organisms. In the case of neurons, the ability of these cells to correctly extend processes to their final synaptic targets is essential for normal development of the nervous system. Although the underlying intracellular pathways involved in migration are much more well-characterized in non-neuronal cell types (Luo et al., 1997; Dickson, 2001; Ridley, 2001; Pollard and Borisy, 2003), certain commonalities have emerged. For instance, the Rho GTPase family of small molecules plays a pivotal role in regulating cytoskeletal dynamics in both neuronal and non-neuronal cell types (Dickson, 2001; Ridley, 2001; Meyer and Feldman, 2002). Rho activation has been shown to induce contraction in yeast and fibroblasts (Ridley and Hall, 1992). Similarly, activation in neuronal populations induces growth cone collapse and retraction (Suidan et al., 1992; Jalink et al., 1994). While such similarities exist, the downstream signaling pathways associated with these molecules have yet to be clearly defined (Luo et al., 1997; Dickson, 2001). Even more poorly understood is whether the mechanisms identified in migrating cells directly translate to similar machinery being

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evoked during neurite outgrowth.

Recently, a novel family of alkaloids, the motuporamines, was isolated from the Papua New Guinea marine sponge, *Xestospongia exigua* (Williams et al., 1998). In a previous study, these motuporamines, particularly isoform C, were found to inhibit cell migration of various human cancer cell lines (Roskelley et al., 2001). Furthermore, it was found that motuporamines attenuate membrane ruffling at the leading edge of lamellae and increase stress fiber formation (Roskelley et al., 2001; McHardy et al., 2004). Since growth cone and non-neuronal cell motility likely require similar cellular rearrangements, I hypothesized that motuporamines could affect growth cone motility in an inhibitory manner, thus allowing the exploitation of this compound as a novel means to study the intracellular pathways that are utilized by neurons for outgrowth.

To investigate this, I utilized motuporamine C (MotC) in an effort to identify which molecular pathways are important for growth cone motility. My results show that MotC acts in an inhibitory, dose-dependent manner. It was found that growth cone collapse occurs within minutes of MotC application, followed by neurite retraction in the continued presence of this compound. These effects are reversible as growth cone motility recovers after MotC removal. Furthermore, I demonstrate that MotC inhibits motility through an upregulation of the Rho-Rho kinase pathway. I have thus shown that this organic molecule, originally identified for its anti-motility effects on cells, is a robust inhibitor of neurite outgrowth. MotC may therefore be utilized as a tool to elucidate key intracellular pathways associated with neurite outgrowth inhibition and collapse.
2.2 Materials and Methods

Motuporamine C collapse assays. 12-well culture dishes were treated overnight at 37°C with 1 µg/ml laminin (Chemicon) and 100 µg/ml poly-L-lysine (Sigma). Explants of embryonic day 8 (E8) chick DRGs and sympathetic neurons were subsequently cultured in these wells with 50 ng/ml NGF (Invitrogen)- or 50 ng/ml NT-3 (Chemicon)-supplemented DMEM (Sigma) overnight at 37°C. Following 24 hour incubation, MotC (dissolved in water) was added to cultures for a further 24 hours (48 hour total). An equivalent volume in microlitres of water was added to control cultures. Washout studies were performed by washing out MotC for 24 hours with pre-warmed NGF-supplemented DMEM media after 24 hour exposure to this compound.

Time-lapse videomicroscopy. Coverslips were coated with 1 µg/ml laminin (Chemicon) and 100 µg/ml poly-L-lysine (Sigma) overnight at 37 °C. E8 DRG explants were cultured on these coverslips containing DMEM supplemented with 20 mM HEPES and 14 mM NaCl overnight at 37 °C. Cultures were then placed on an inverted Nikon DIAPHOT 200 fluorescence microscope, with time-lapse imaging captured using a Princeton Instruments MicroMax CCD camera (Kodak chip KAF 1400) and MetaView Imaging System 3.6 (Universal Imaging Corporation). Cultures were filmed for 1 hour in the absence of MotC, followed by 1 hour in its presence. For time-lapse of washout, MotC was washed out with DMEM supplemented with 20mM HEPES and 14 mM NaCl and filmed for another 3 hours.

Immunocytochemistry. Cultures were fixed with 3.7% formaldehyde in PEM (0.1 M PIPES, 2 mM EGTA, 1 mM MgSO₄) for 10 min, and then washed with PBS containing
0.1% Triton-X and 0.1% BSA (3 x 1 minute, followed by 3 x 10 minutes). Rabbit-anti-neurofilament (Sigma) primary antibody (1:500) was applied overnight (4°C) and then washed again in PBS containing Triton X-100 and BSA as described above. Cells were then incubated at room temperature in 1:500 Cy3-conjugated goat-anti-rabbit (Jackson Laboratories) secondary antibody.

Growth cone assay. DRG explants were cultured for 24 hours as described above, followed by 1 hour exposure to MotC. Explants were subsequently washed with PBS (2 x 1 minute), then fixed with 3.7% formaldehyde solution in PBS for 10 minutes at room temperature. After fixation, cultures were washed in PBS (2 x 1 minute), once for 5 minutes in PBS containing 0.1% Triton X-100, followed by PBS (2 x 1 minute) once more. For actin staining, explants were then stained with 2 units of Alexa Fluor 488 phalloidin (Molecular Probes) diluted into 200 µl PBS containing 1% BSA for 30 minutes. For microtubule staining, explants were fixed using a microtubule stabilizing fixative (Dent et al., 1999). This fixative was 4% paraformaldehyde / 0.25% glutaraldehyde containing 0.1% Triton X-100, 10µM taxol, and 1.3µM phalloidin in PHEM buffer. Following a 15 minute fixation, neurons were washed in PBS and then incubated in mouse-anti-acetylated tubulin (Sigma) and rat-anti-tyrosinated tubulin antibodies for 1 hour at room temperature and then washed again in PBS containing Triton X-100 and BSA as described above. Cells were then incubated at room temperature in 1:500 Cy3-conjugated goat-anti-mouse (Jackson Laboratories) and 1:500 Alexa 488 goat-anti-rat secondary antibodies.

Rho-GTP Affinity Precipitation and Immunoblot. DRG explants were dissected from E8 chicks. Using the Rho activation assay kit (Upstate), pull-down of Rho-GTP was
performed as described. Briefly, experimental lysates (1 mg/ml) were treated with MotC for 1-3 hours at 37°C before the addition of Rhotekin Rho-binding domain agarose beads for 45 minutes at 4°C. The slurry of beads were then boiled for 5 minutes with 2x Laemmli reducing buffer and 1M dithiothreitol, and then loaded into a 15% polyacrylamide gel at which point SDS-PAGE was performed using a 1:333 anti-Rho primary antibody (Upstate) and 1:1000 goat-anti-rabbit HRP conjugated IgG secondary antibody (Jackson Laboratories). An ECL-based detection protocol was used for protein identification. Equal protein loading levels were determined using a colorimetric protein assay kit (BioRad) and a Western blot detecting total levels of Rho protein in lysates. These pull-downs were done in triplicate.

**ROCK Inhibition assay.** DRG explants were cultured for 24 hours as described above. Following overnight incubation, the ROCK inhibitor Y27632 (Calbiochem) was added 1 hour prior to MotC addition, and both compounds incubated with these cultures for another 24 hours (48 hour total). Immunocytochemistry or phalloidin stain was then performed as described above.

**C3 Exoenzyme Loading.** E8 chick DRG explants were dissociated and triturated with C3 exoenzyme as previously described (Jin and Strittmatter, 1997). Briefly, explants were isolated and then re-suspended in 25 mM Tris-HCl, 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, and 5-20 µg/ml C3 exoenzyme from *Clostridium botulinum* (Calbiochem). This suspension was then passed 50 times through an Eppendorf P200 pipette tip and then plated in 20 volumes of DMEM media supplemented with 50 ng/ml NGF (Invitrogen). Cultured neurons were allowed to grow for 24 hours, followed by application of 5 µM MotC for 1 hour in the case of the experimental condition, at which
point neurons were fixed and stained with Alexa Fluor 633 phalloidin (Molecular Probes) as described above.

Quantification. Slides were viewed using a Nikon DIAPHOT 200 inverted fluorescence microscope, with images of neurites and growth cones captured using a cooled CCD camera (Princeton Instruments) and MetaView imaging software (Universal Imaging Corporation). Neurite length was measured by recording radially 1 in every ~50 neurites per explant. Each experiment was done in triplicate with 15 explants per condition. Microsoft Excel was used to calculate the standard error of mean and standard deviation for each experimental condition. Statistical significance of differences between conditions was determined by ANOVA and Student’s t-test using SPSS statistical software version 11.0 for Windows. For quantification of growth cone collapse, growth cones were scored as collapsed according to Fan et al. (1991). Specifically, growth cones lacking lamellipodia and extending 4 or less filopodia were scored as collapsed.
2.3 Results

**MotC inhibits neurite outgrowth and stimulates growth cone collapse**

To examine the effects of MotC on neurite outgrowth, I cultured embryonic day 8 chick dorsal root ganglion (DRG) explants overnight in culture media containing NGF and subsequently added varying concentrations of MotC. Following a 24 hour exposure to this compound, I found that as MotC concentrations were increased, neurite outgrowth decreased significantly in a dose-dependent manner (Fig. 2.1). Initial baseline concentrations of MotC were determined from previous studies carried out by Roskelley et al. (2001) on non-neuronal cultures. At the lower concentrations, neurite outgrowth of DRG's appeared to halt. At moderate to high amounts, neurite retraction likely occurred since neurite length following 24 hour MotC addition (48 hour total) was shorter than that at 24 hours initial growth (data not shown). A stepwise increase in concentration from 5 μM to 6 μM resulted in the most significant decrease in outgrowth. At concentrations higher than 7 μM MotC, I found cultured DRGs could not adhere to the tissue culture substrate following exposure to this compound. I observed that embryonic day 8 dissociated DRG cultures and sympathetic neurons responded in a similar inhibitory manner to MotC (data not shown).

Observations of bleb-like structures at neurite endings following addition of MotC suggested that MotC may cause growth cone collapse. Growth cone collapse usually involves the loss of the actin cytoskeleton integrity in the filopodia and lamellipodia (Jin and Strittmatter, 1997). I examined whether MotC was specifically causing growth cone
Figure 2.1. Inhibitory effects of MotC on E8 DRG explants.

(A) Control explant cultured for 48 hours in culture medium supplemented with NGF. (B-D) Explants cultured for 24 hours in culture medium supplemented with NGF, followed by a further 24 hours in DMEM supplemented with NGF and either 4, 5, or 6 μM MotC, respectively. (E) Quantification of the effects of MotC on DRG neurite outgrowth. The percentage of neurites longer than a given length was plotted versus neurite length. For each explant of each condition, a measurement of neurite length was taken every ~50 neurites radially along the explant. n=50 neurite length measurements from 15 DRGs per experimental condition, with experiments done in triplicate. Scale bar represents 100 μm.
Neurite Length (µm) = x

% Neurites > x:

- 120.0%
- 100.0%
- 80.0%
- 60.0%
- 40.0%
- 20.0%
- 0.0%

Neurite Length (µm) = x

- 0µM MotC
- 3µM MotC
- 4µM MotC
- 5µM MotC
- 6µM MotC
- 7µM MotC
collapse by depolymerizing the actin cytoskeleton. I found that following 1 hour application of MotC, fluorescent phalloidin labeling showed complete collapse of the actin network in growth cones at all concentrations tested (ranging from 2 μM to 7 μM) (Fig. 2.2B), while control cultures maintained their lamellipodial and filopodial processes (Fig. 2.2A). I next analyzed whether MotC had any effects on the microtubule organization in the growth cone (Fig. 2.2C,D). By labeling both the tyrosinated and acetylated tubulin populations, I found in control neurons, a predominance of the dynamic tyrosinated microtubules are observed in the central domain and near the leading edge of the growth cone, while the acetylated population was primarily found along the neurite shaft and at the base of the growth cone where there was an overlap with the tyrosinated microtubules (Fig. 2.2C). This distribution of tubulin supports previous observations of microtubule organization (Robson and Burgoyne, 1989; Fan et al., 1993). In contrast, growth cones examined following 60 minutes of MotC treatment showed a loss of much of the dynamic tyrosinated microtubule network and a much larger region of overlap between the tyrosinated and acetylated microtubule populations (Fig. 2.2D). However, a core of tyrosinated microtubules remained after treatment suggesting that MotC-induced growth cone collapse results in a disassembly of tyrosinated microtubules and a possible retraction into the acetylated microtubule domain (Fig. 2.2D). These results show that MotC induces collapse through an actin-dependent mechanism and may also contribute to the disassembly of the dynamic microtubule population at the leading edge of the growth cone.
Figure 2.2. Growth cone morphology in response to MotC.

(A) Phalloidin-stained control DRG growth cones cultured for 25 hours. (B) Phalloidin-stained growth cones cultured for 24 hours, followed by 1 hour in media supplemented with 5 μM MotC. (C) Control growth cones immunostained for tyrosinated microtubules (green) and acetylated microtubules (red). (D) A growth cone cultured for 24 hours under normal conditions, followed by 1 hour in media supplemented with 5 μM MotC, and immunostained for tyrosinated (green) and acetylated microtubules (red). Solid arrows indicate examples of regions of overlapping tyrosinated and acetylated tubulin. Scale bar represents 10 μm.
The effect of MotC is reversible

To address if the effects of MotC are reversible and/or cytotoxic, I conducted washout experiments. DRGs were cultured for 24 hours, exposed to MotC for 24 hours, and then cultured an additional 24 hours in the absence of MotC. In these explants, I observed neurite outgrowth recovery up to concentrations of 5 μM MotC (Fig. 2.3A-D). In addition, the recovery length during washout consistently reached approximately 50% of the outgrowth that occurred in the first 48 hours of the experiment (Fig. 2.3E). At concentrations higher than 5 μM, neurons did not resume outgrowth following removal of MotC. These results show that the inhibitory effects of moderate levels of MotC are reversible.

To confirm these observations I used time-lapse videomicroscopy to directly observe MotC-induced growth cone collapse and neurite recovery following removal of MotC (Fig. 2.4). Initial time-lapse imaging in normal media showed growth cones actively sampling the environment via extending and retracting filopodial processes (Fig. 2.4A). Within 60 minutes of MotC addition, complete collapse was observed, with the initial collapse response being observed within the first 10 minutes of addition (Fig. 2.4B-C). The initial responses included withdrawal of filopodial processes back towards the growth cone and lamellipodial veil retraction. In addition, I observed bleb-like structures retracting back towards the cell body, resulting in axon blebbing. Further examination suggests that these bleb-like structures are, at least in part, collections of disassembled actin. When MotC was removed after 75 minutes of exposure, normal growth cone morphology was re-established, presumably by actin and microtubule re-
Figure 2.3. Removal of MotC allows DRG explants to recover.

(A-B) Control explant cultured for 48 hours, followed by a further 24 hours in fresh control medium. (C-D) DRG explants in which explants are cultured for 24 hours in absence of MotC, followed by a further 24 hours in the presence of 4 µM MotC (C), at which point MotC is removed and replaced with control medium for a final 24 hours (D). (E) Quantification of average neurite length of each condition prior to and following washout. n=50 neurite length measurements from 15 DRGs per experimental condition, with experiments done in triplicate. Error bars=standard error of the mean. * p < 0.01. Scale bar represents 100 µm.
**Figure 2.4.** Timelapse videomicroscopy confirms collapsing effect and reversibility of MotC.

(A) Embryonic day 8 chick growth cones cultured in normal media for 1 hour. (B) Initial time point in which MotC was added to media. The black arrow indicates the presence of MotC in media. (C) Following 1 hour of MotC application, growth cones have completely collapsed. (D) Final time point in which MotC was present just prior to washout. (E-F) Washout of MotC results in the functional recovery of growth cones, as filopodia re-extend into the surrounding environment. White arrow indicates growth cone, while black arrowhead demarcates filopodial process. Scale bar represents 100 μm.
assembly. Furthermore, I found that filopodia were able to re-extend back into the periphery and resume active sampling of the environment, indicating growth cone motility was re-established and that the effects of this compound on growth cone dynamics are reversible (Fig. 2.4E-F).

The effects of MotC are neurotrophin independent

Nerve growth factor (NGF) has been shown to promote survival and growth of neurons through its high-affinity TrkA receptor on the plasma membrane (Kaplan et al., 1991). Since the media was supplemented with NGF to promote outgrowth of DRGs in the experiments described above, it was possible that the observed effects of MotC were due to the attenuation of the signaling mechanisms associated with this particular growth factor. Therefore, an alternate growth factor, neurotrophin-3 (NT-3), was also used. Recent evidence has suggested NGF and NT-3, acting through their TrkA and TrkC receptors, respectively, can elicit their effects through distinct signaling pathways (Song and Poo, 1999; Patapoutian and Reichardt, 2001). Furthermore, it has been shown by Hory-Lee et al. (1993) that both NGF and NT-3 support different chick sensory neuron populations. In mouse studies, mutants null in individual Trk receptors lack specific subpopulations of DRG neurons (Fagan et al., 1996; Canover and Yancopoulos, 1997). In TrkA nulls, mice lack nociceptive and thermoreceptive neurons, while TrkC nulls lack proprioceptive neurons (Fagan et al., 1996; Canover and Yancopoulos, 1997). Therefore, NT-3 was used to address whether MotC was only inhibiting growth of a subpopulation of DRG neurites. Chick DRGs were cultured in DMEM media supplemented
with NT-3 instead of NGF, followed by a further 24 hour incubation in the presence of MotC. In this assay, I found MotC induced similar, dose-dependent inhibitory effects (Fig. 2.5) when compared to the outgrowth inhibition assays on NGF. Though NT-3 cultured DRGs have less outgrowth in control conditions as compared to control DRGs cultured with NGF, the average percent inhibition of outgrowth by MotC between these two culture conditions is approximately equal (data not shown). These data suggest that MotC does not act by attenuating the signaling of a specific growth factor pathway. Rather, it suggests the activation of a common downstream pathway as culturing neurons with different growth factors that stimulate different signaling pathways for outgrowth, MotC attenuates outgrowth in both conditions in an analogous manor.

**MotC stimulates Rho activation**

Previous studies have shown that the small GTPase Rho plays a central role in growth cone collapse and retraction (Sebok et al., 1999; Lehmann et al., 1999). Moreover, recent evidence has shown an upregulation of the Rho-GTP pathway in Swiss-3T3 fibroblasts in response to MotC addition (McHardy et al., 2004). Therefore, I examined whether this pathway was also induced in DRG explants treated with MotC via a rhotekin-based pull-down assay. Neurons treated with MotC for 1-3 hours showed an increase in the levels of active Rho-GTP of approximately 25% (determined by densitometry) as compared to control neurons (Fig. 2.6A), suggesting that Rho-mediated signaling is responsible for growth cone collapse. To examine this further, I utilized the C.
Figure 2.5. Inhibitory effect of MotC is independent of growth factor used to culture DRG explants.

(A) Control explant cultured for 48 hours in culture medium supplemented with only NT-3. (B) Explants cultured for 24 hours in medium supplemented with only NT-3, followed by a further 24 hours in medium supplemented with NT-3 and 5 μM MotC. (C) Quantification of the effects of MotC on DRG neurite outgrowth. The percentage of neurites longer than a given length was plotted versus neurite length. For each explant of each condition, a measurement of neurite length was taken every ~50 neurites radially along the explant. n=50 neurite length measurements from 7 DRGs per experimental condition, with experiments done in triplicate. Scale bar represents 100 μm.
Neurite Length (um) = x

A: 0μM MotC
B: 5μM MotC

C: Graph showing the percentage of neurites greater than a certain length (x), with different concentrations of MotC.
Figure 2.6. Application of MotC activates the Rho pathway.

(A) Rho-GTP affinity precipitation and immunoblot analysis using Rhotekin Rho-binding domain agarose beads. In control conditions (left lane), DRGs were incubated for 1-3 hours prior to lysing, whereas in experimental conditions (right lane), DRGs were incubated in the presence of 5 μM MotC prior to lysing. For each condition, 1 mg/ml of DRG lysate was used. (B) Phalloidin-stained dissociated DRG neurons triturated with 0 μg/ml C3 exoenzyme cultured for 24 hours, followed by subsequent addition of 5 μM MotC for 1 hour. (C) Dissociated DRG neurons triturated with 5 μg/ml C3 exoenzyme, followed by addition of 5 μM MotC for 1 hour. (D) Quantification of growth cone collapse expressed as percent collapsed. n=60 growth cones scored per experimental condition, with experiments done in triplicate. Error bars=standard error of the mean. ** p < 0.01 as compared to MotC alone treatment. Scale bar represents 10 μm.
botulinum-derived inhibitor, C3 exoenzyme, to inhibit all three Rho isoforms. Via crystal structure analysis, Han et al. (2001) showed that C3 specifically inhibits Rho via ADP ribosylating asparagine-41. Furthermore, Jin and Strittmatter (1997) showed that neither constitutively active forms of Rac or Cdc42 reversed the inhibitory actions of C3 transferase on Rho in chick DRGs, providing further evidence of C3’s specificity to Rho. In this study, neurons that did not contain the C3 exoenzyme and were applied MotC resulted in collapse of 90% of the growth cones within 1 hour (Fig. 2.6B,D). In contrast, neurons treated with C3 exoenzyme (by tritration) exhibited a significant reduction in growth cone collapse (Fig. 2.6C,D). Experiments repeated with a higher C3 exoenzyme concentration (20μg/ml) showed no additional inhibition of MotC-induced growth cone collapse. These results show that a pan-Rho inhibitor is able to partially rescue the MotC-induced collapse phenotype.

Since previous studies have shown that Rho kinase (ROCK) acts as a major downstream effector of activated Rho, I next examined whether ROCK activity was required during MotC-mediated growth inhibition. To test this, I inhibited the Rho downstream signaling kinase, ROCK, to see if MotC-induced growth cone collapse would be attenuated. The ROCK inhibitor Y27632 has previously been shown to be a competitive inhibitor for the ATP binding site of ROCK (Yamaguchi et al., 2006). Furthermore, Y27632 displayed a selectivity profile towards ROCK in an experiment that compared its binding to 25 protein kinases (Davies et al., 2000). In the absence of MotC, neurons cultured in the presence of Y27632 showed no phenotypic difference as compared to wild type control conditions (Fig. 2.7A,B). However, when DRG neurons
Figure 2.7. Morphological changes to the growth cone in response to MotC and the ROCK inhibitor, Y27632.

(A) Phalloidin-stained control DRG growth cones cultured for 25 hours in media. (B) Control DRG growth cones cultured for 23 hours in media, followed by 2 hours in media supplemented with 30 μM Y27632. (C) Growth cones cultured for 24 hours, followed by 1 hour application of 5 μM MotC. (D) Growth cones cultured for 23 hours in media, followed by 2 hours in media supplemented with 30 μM Y27632, and subsequently followed by a further 1 hour in media supplemented with 30 μM Y27632 and 5 μM MotC. Solid arrow indicates example of collapsed growth cone, while outlined arrow indicates example of normal, active growth cones. Scale bar represents 7 μm.
Figure 2.8. Outgrowth inhibition by MotC requires the downstream Rho effector, ROCK.

(A-C) Control condition in which explants were cultured for 24 hours, followed by a further 24 hours with no addition (A) or with addition of 30 μM (B), or 60 μM Y27632 (C). (D-F) Experimental condition in which explants were cultured for 24 hours, followed by a further 24 hours in 5 μM MotC with either 0 μM (D), 30 μM (E), or 60 μM Y27632 (F). (G) Quantification of average neurite length of each condition in the absence and presence of 30 μM Y27632. n=50 neurite length measurements from 15 DRGs per experimental condition, with experiments done in triplicate. Error bars=standard error of the mean. * p < 0.01. Scale bar represents 100 μm.
were cultured in the presence of MotC and Y27632, it was found that growth cone collapse was partially prevented at MotC concentrations 5 μM and lower (Fig. 2.7C, D and data not shown). At these concentrations, explants were observed to exhibit some collapsed growth cones, as well as normal non-collapsed growth cones. Since MotC-mediated growth cone collapse was attenuated, I examined neurite outgrowth in the presence of both MotC and Y27632. DRGs were cultured for 24 hours and then either 30 μM or 60 μM Y27632 was added 1 hour prior to the addition of MotC, at which point cultures were incubated with both inhibitors for a further 24 hours. Initially, both 30 μM and 60 μM Y27632 levels were used to ensure the maximal effective concentration was used (Fig. 2.8A-F).

Quantitatively, I found no significant difference between 30 μM and 60 μM Y27632 (data not shown), confirming previous observations with neurons grown on inhibitory substrates (Borisoff et al., 2003). I therefore used the 30 μM concentration for the remainder of the experiments. I found DRGs receiving both ROCK inhibitor and MotC had an average increased neurite length of 60% as compared to DRGs receiving only MotC (Fig. 2.8G). To control for the fact that inhibiting the Rho-ROCK pathway would allow greater neurite outgrowth even in the untreated cultures, I cultured DRGs with only Y27632 and no MotC. Under these conditions, I found explants had an increased neurite length of approximately 30% as compared to DRGs receiving no inhibitors at all. Therefore the effects of Y27632 are due in part to the inhibition of MotC signaling. Also, as only 60% recovery of neurite length was observed, this suggests that additional pathways may also be important for collapse. Thus MotC may prove to be a
useful tool to identify the full complement of signaling pathways important for growth cone collapse and neurite retraction.
2.4 Discussion

In this study, I have found that the anti-motility compound, MotC, is a robust inhibitor of neurite outgrowth and can stimulate growth cone collapse. This confirms previous observations that cell and growth cone motility may share several properties including signaling pathways. Thus I have utilized this compound to develop a robust, inhibitory assay to examine the intracellular mechanisms involved in neurite outgrowth and retraction. I demonstrate that MotC acts as a reversible, dose-dependent inhibitor of neurite outgrowth for multiple neural populations. Furthermore, inhibition is initiated via growth cone collapse within minutes of MotC application, followed by neurite retraction. Due to the relatively quick response of growth cones to MotC, it is likely that it is inducing a localized effect on the cytoskeleton in the growth cone. Furthermore, cytoskeletal staining indicates remodeling of both the actin and microtubule networks during MotC-mediated collapse. In the case of microtubules, much of the dynamic microtubule population disassembles (although not completely) while the stable population appears unaffected. The actin network is more severely impacted with complete loss of F-actin in the filopodia and leading edge. I also observed bleb-like structures associated with neurites exposed to MotC. Rhodamine phalloidin staining suggests that these structures are made up of, at least in part, short F-actin filaments. Future experiments will be required to determine whether the disassembly of the microtubule network is a secondary effect due to F-actin loss or independently regulated.

My data also suggest that MotC is likely not attenuating a specific growth-promoting pathway and not specifically inhibiting the outgrowth of one sub-population of
neurites. Rather, MotC is a robust growth-inhibiting compound that is acting, at least in part, by upregulating the Rho pathway. It has been established that the Rho family of GTPases play a central role in the actin dynamics that drive growth cone motility (Luo et al., 1997; Dickson, 2001). Similarly in the case of Rho, it has been found that its activation results in inhibition and/or retraction of outgrowth in a number of cell types, including Xenopus tectal neurons, PC12 neurites, and rat retinal ganglion cells (Sebok et al., 1999; Lehmann et al., 1999; Lee et al., 2000; Li et al., 2000). In agreement with these studies, I find that MotC induces inhibition of neurite outgrowth via the Rho-ROCK pathway. However, I find that the C3 exoenzyme and ROCK-inhibiting experiments only partially attenuate the effects of MotC, both at the level of growth cone structure as well as total neurite length, suggesting two possible scenarios. The first is that MotC may be modulating other signaling pathways associated with outgrowth. This is supported by the fact that the use of higher concentrations of both Y27632 and C3 exoenzyme inhibitors did not further attenuate the effects of MotC. In the case of ROCK inhibition by Y27632, a previous study by Borisoff et al. (2003) showed that a peak response to this inhibitor occurred between 25-50 μM. Using a similar concentration range I found no increase in attenuation between 30-60 μM. Similarly, increasing C3 exoenzyme concentrations did not further attenuate the effects of MotC, supporting the suggestion that alternative pathway(s) could be involved. Such pathways include those downstream of other Rho GTPase family members. For example, despite observing an upregulation in Rho-GTP activity, one cannot exclude the possibility of a concomitant change in regulation of Rac or Cdc42 activity. Since previous studies have found that increased activity of both Rac and Cdc42 is associated with increased cell migration (Ridley and Hall, 1992; Nobes and
Hall, 1995) and neurite outgrowth (Threadgill et al., 1997; Brown et al., 2000), one could speculate that MotC may be downregulating these two Rho GTPases. Future studies will be necessary to dissect the intracellular complexities associated with MotC. A second scenario is the possibility that the inhibitors used to block the Rho-ROCK pathway are not completely specific. Future experiments to address such caveats are required.

The effects of this novel compound are similar to other inhibitory compounds normally associated with growth cone collapse and outgrowth inhibition. For example, the phospholipid, lysophosphatidic acid (LPA), has been shown to produce a wide variety of responses in many cell types, ranging from growth cone collapse in retinal neurons and DRGs to cell proliferation in astrocytes (Van Corven et al., 1989; Ye et al., 2002). Despite this broad response, the collapsing effect of LPA induces actin-mediated collapse through the RhoA pathway (Ye et al., 2002). Another inhibitory protein able to induce MotC-like effects is the semaphorin guidance molecules, particularly the semaphorin-3 family, which has been shown to induce collapse at the leading edge and act as a repulsive guidance cue (Luo et al., 1993). After semaphorin-3A exposure, growth cones rapidly undergo filopodial contraction and actin-mediated collapse (Jin and Strittmatter, 1997; Luo et al., 1993). In addition, semaphorins have been shown to signal through their plexin and neuropilin receptors to directly associate with the Rho-GTPases (Jin and Strittmatter, 1997; Takahashi et al., 1999; Vastrik et al., 1999). Interestingly, in the case of Sema3A/PlexinA1-induced growth cone collapse, it has previously been shown that the activity of Rac, rather than RhoA, is required (Jin and Strittmatter, 1997). This suggests intracellular pathways other than RhoA can mediate growth cone collapse.
Previous studies have shown MotC to have anti-invasive and low cytotoxic effects (Roskelley et al., 2001). Recently, McHardy et al. (2004) have shown in non-neuronal cells that MotC activates Rho-GTP and stimulates Rho kinase-dependent sodium proton exchanger activity. My results are similar in that neurite outgrowth is inhibited by MotC via an upregulation of Rho kinase activity. Further experiments will be necessary to determine if this inhibition is due in part to a stimulation of sodium proton exchanger activity. In addition, McHardy et al. (2004) have also shown a marked rise in staining of the adhesion complex marker, vinculin, following MotC application. This suggests a role for adhesion in mediating the non-motile effects of MotC and will require future examination.

These lines of evidence point to the suggestion that at least some of the mechanisms are conserved between neurite outgrowth and cell migration. Future studies identifying whether alternate pathways are also involved will be critical in gaining further insight into the molecular machinery involved in growth cone motility in an inhibitory environment. Since MotC exhibits robust activity, is not subject to proteolysis, and is easy to use, it serves a valuable tool to further elucidate these intracellular mechanisms.
2.5 References


3. MOTUPORAMINE C ACTIVATES MULTIPLE CONCURRENT PATHWAYS TO STIMULATE GROWTH CONE COLLAPSE

3.1 Introduction

During normal development, extending axons must correctly navigate through the extracellular environment to make functional connections with their end targets. In order to do this, the motile tip of the axon must decode signals from the environment into information required for motility. How these extracellular signals are translated into this directional movement remains poorly understood. The elucidation of such intracellular mechanisms associated with motility may be advantageous to promote functional recovery following some types of neuronal injury.

One well-characterized effector molecule associated with outgrowth inhibition is the Rho GTPase family member Rho in which activation in neurons results in growth cone collapse and neurite retraction (Jalink et al., 1994; Meyer and Feldman, 2002; To et al., 2006). Recently, I have characterized a novel stimulator of outgrowth inhibition, termed Motuporamine C (MotC), that stimulates robust growth cone collapse and neurite retraction via upregulation of active Rho-GTP and its downstream target, Rho kinase (ROCK) (To et al., 2006). However, I observed MotC-mediated collapse could only be partially attenuated by blocking the Rho-ROCK pathway, suggesting other pathways are

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also involved. Despite the strong evidence for active Rho playing an inhibitory role in growth cone motility, it remains poorly investigated what other signal transduction pathways are involved during this process.

One area of focus has been the identification of Ca\(^{2+}\) as a mediator of neuronal motility. Numerous guidance cues have been identified that depend on Ca\(^{2+}\) signaling in growth cones, including nerve growth factor (NGF) (Gundersen and Barrett, 1980), brain-derived neurotrophic factor (BDNF) (Song et al., 1997), netrin-1 (Hong et al., 2000), and myelin-associated glycoprotein (MAG) (Henley et al., 2004). Interestingly, addition of the chemoattractants netrin-1 and BDNF to cultured cerebellar granule cells has been shown to upregulate activity of the Rho GTPase family members Rac and Cdc42 via Ca\(^{2+}\) influx (Jin et al., 2005). Furthermore, netrin-1 has been shown to induce a mid-amplitude rise in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) via influx through plasma membrane Ca\(^{2+}\) channels and Ca\(^{2+}\) release from internal stores to promote attractive turning of growth cones in *Xenopus* spinal neurons (Hong et al., 2000). In contrast, the chemorepellent MAG has been shown to produce a low-amplitude [Ca\(^{2+}\)]\(_i\) rise in the same cell types through Ca\(^{2+}\) release from internal Ca\(^{2+}\) stores alone (Henley et al., 2004).

Recent examination of other downstream targets of Ca\(^{2+}\)-mediated growth cone motility have identified the cysteine protease calpain to also play an important role. In non-neuronal cells, inhibition of calpain has been shown to impair retraction of the trailing edge and therefore reduce migration, suggesting a role for this Ca\(^{2+}\)-sensitive protease in promoting adhesion complex turnover (Franco et al., 2004; Palecek et al., 1998). In contrast, using *Xenopus* spinal neurons, Robles et al. (2003) has shown that the
repulsive effects of asymmetrical high $\text{Ca}^{2+}$ transients directly induced on one side of the growth cone activates calpain locally, and inhibition of calpain attenuates these inhibitory effects on motility. This suggests calpain may play an opposite role in neurons as compared to other cell types, although no study to date has examined calpain involvement during extracellular cue-induced regulation of growth cone motility. It is important to note that while growth cones do not contain focal adhesions per se, focal contacts appear to serve an equivalent role. Proteins found associated with focal contacts include integrins, kinases, phosphatases, and the actin-associated proteins talin and vinculin (Gomez et al., 1996; Letourneau and Shattuck, 1989; Schmidt et al., 1995).

Based on the data supporting $\text{Ca}^{2+}$ involvement in motility, I examined whether $\text{Ca}^{2+}$ was involved in MotC-mediated growth cone collapse. MotC is an organic molecule not endogenously found in vertebrates. Originally developed as a potential therapeutic in cancer due to its anti-motility effects in a number of cancer cell lines (Roskelley et al., 2001), I have previously characterized MotC as a potent collapse of growth cones (To et al., 2006). Based on its robust activity and ease of use, MotC can serve as a valuable research tool to further identify intracellular pathways involved in growth cone collapse. In this study, I report MotC induces a high-amplitude increase in $[\text{Ca}^{2+}]$, via $\text{Ca}^{2+}$ influx from the extracellular environment. This $\text{Ca}^{2+}$ increase results in upregulation of calpain activity. Furthermore, I present evidence that calpain- and Rho-ROCK-mediated pathways act in combination to induce growth cone collapse. To the best of my knowledge, this is the first study to show concurrent activation of calpain and Rho-ROCK signaling during a growth cone collapsing event, and underscores the complexity of multiple downstream targets being activated during a motile response.
3.2 Material and Methods

Inhibitors and antibodies. MotC was isolated as previously described (Roskelley et al., 2001) and used at a concentration of 4 μM. Stock Fura-2-AM (Invitrogen) was dissolved in chloroform at 1 mg/ml, vacuum evaporated to boil off chloroform, and stored in -80°C for long-term use. EGTA and cobalt was purchased from Fisher Scientific and prepared fresh for each experiment at 1 mM and 50 mM, respectively. Cyclosporin A (CsA) and Deltamethrin (DM) were purchased from Calbiochem and prepared as previously described (Lautermilch and Spitzer, 2000). Briefly, CsA and DM were prepared fresh by dissolving in a 1:1 mixture of ethanol and DMSO warmed to 37°C, allowed to cool, and then used immediately at 50 nM and 5 nM, respectively. PD150606 and ALLM (Calbiochem) were dissolved in DMSO to be used at 30 μM and 10 μM working concentrations, respectively. 7-amino-4-chloromethylcoumarin, t-BOC-L-leucyl-L-methionine amide (t-BOC) (Invitrogen) was dissolved in DMEM and used at a working concentration of 5 μM.

Preparation of cell cultures. Lumbar explants of embryonic day 10 (E10) White Leghorn chick DRGs were cultured on 18 mm glass coverslips (Fisher Scientific) in 12-well culture dishes (Falcon) which were treated overnight with 1 μg/ml laminin (Chemicon) and 100 μg/ml poly-L-lysine (Sigma). DRGs were cultured in these wells containing DMEM (Sigma Aldrich) supplemented with 50 ng/ml 7S-NGF (Invitrogen) for 24 hours at 37°C. Dissociated DRG cultures were prepared as previously described (Nakamoto and Shiga, 1998) and similarly cultured for 24 hours at 37°C.

Calcium imaging. Using the dual-excitation ratio method as previously described (Church et al., 1998), measurements of \([\text{Ca}^{2+}]\), were made on an imaging system.
(Northern Eclipse, Empix Imaging Inc.) paired to a Zeiss Axioskop FS Plus microscope with an Achroplan 100x water immersion objective (Carl Zeiss Canada). Briefly, dissociated DRG cultures were incubated with 5 μM Fura-2-AM diluted in DMEM without phenol red (Sigma Aldrich) and containing 0.02% Pluronic F-127 (Calbiochem) for 45 minutes, and then washed in DMEM without phenol red for 15 minutes. Cultures were then placed in a temperature-controlled recording chamber (Warner Instruments) set at 37°C and supplied with 5% CO₂, 21% O₂, and balanced N₂. Fluorescence emissions measured at 510 ± 20 nm from regions of interest placed on individual growth cones of fura-2-loaded neurons were acquired by a cooled CCD camera (Retiga EXi, QImaging). Ratio pairs of excitation wavelength 340 and 380 nm were acquired at 10 second intervals with background fluorescence corrected for. Normalized ratio values of relative changes in [Ca²⁺]ᵢ were calculated by dividing the change in fura-2-derived ratio values induced by an experimental stimulus by the ratio value just prior to this change in conditions, and averaged from 15 growth cones per condition (expressed as Rₙ). In the case of Ca²⁺-free experiments, [Mg²⁺] was increased to 1.8 mM, and 1 mM EGTA was added. In the case of ROCK inhibition experiments, 30 μM Y27632 was added to the media.

**MotC growth cone collapse assays.** Following 24 hours incubation at 37°C to allow normal outgrowth, MotC was added to cultures for 1 hour at 37°C. For conditions in which inhibitors to downstream MotC targets were used, a 20 minute pre-incubation with these inhibitors was employed. Following 1 hour incubation, cultures were fixed with 3.7% formaldehyde solution in PBS for 15 minutes at room temperature, washed in PBS (2 x 5 minutes), then 5 minutes in PBS containing 0.1% Triton X-100, and subsequently
by PBS (2 x 5 minutes) once more. Growth cone morphology was examined using 2 units of Alexa Fluor 488 phalloidin (Invitrogen) diluted into 200 µl PBS containing 1% BSA per well for 30 minutes.

*t-BOC calpain activity assay.* Dissociated cultures were used to allow for more uniform uptake of the fluorescence calpain activity reporter molecule, *t*-BOC (Invitrogen), and thus provide clearer visualization of activity along neurites. *t*-BOC was utilized in these cultures under the following conditions: control cultures, cultures pre-incubated with 30 µM PD150606 for 20 minutes, cultures incubated with MotC for 30 minutes, and cultures pre-incubated with 30 µM PD150606 for 20 minutes followed by MotC addition for 30 minutes. *t*-BOC was added 10 minutes prior to visualization of calpain activity in all experimental conditions. Fluorescent imaging of calpain activation was determined under live cell conditions.

*Quantification.* The criteria of growth cones scored as collapsed was defined as previously described by Fan at al. (1993). Briefly, growth cones lacking lamellipodia and extending 4 or less filopodia were considered collapsed. Experiments were done in triplicate with 50 growth cones scored per condition. Quantification of calpain activity with *t*-BOC was determined by measuring the average fluorescent intensity of 20 growth cones per condition and dividing by baseline control intensities. SPSS statistical software version 11.0 for Windows was used to determine standard error of mean, standard deviation, and statistical significance between conditions via ANOVA and Student’s *t*-test.
3.3 Results

MotC induces a marked rise in $[\text{Ca}^{2+}]_{i}$ during growth cone collapse

To examine whether $\text{Ca}^{2+}$ mediates the growth cone collapsing effects of MotC, I utilized Fura-2-AM fluorescence ratiometric imaging techniques to measure changes in $[\text{Ca}^{2+}]_{i}$ (Fig. 3.1). Following MotC addition, intracellular $\text{Ca}^{2+}$ levels in growth cones were characterized by an initial latency period of 6 minutes ($\pm$ 1 minute; $n = 15$ growth cones per condition), followed by a sudden high-amplitude increase in $[\text{Ca}^{2+}]_{i}$ which was sustained for the remainder of the experiment (Fig. 3.1A-D). Previously, I have shown significant morphological changes associated with MotC-mediated growth cone collapse occurs within 15-20 minutes of MotC application (To et al., 2006). The observed increase in $[\text{Ca}^{2+}]_{i}$ thus precedes these morphological changes, suggesting $\text{Ca}^{2+}$ may be responsible for the initiation of this event. In contrast, parallel control cultures exhibited a constant basal level of $[\text{Ca}^{2+}]_{i}$ and no growth cone collapse was observed (Fig. 3.1D).

Since the chemoattractant netrin-1 has previously been shown to induce a mid-amplitude rise in $[\text{Ca}^{2+}]_{i}$ and that this specific amplitude rise is associated with outgrowth (Gomez and Zheng, 2006; Hong et al., 2000; Kater and Mills, 1991), I examined netrin-1-mediated regulation of $[\text{Ca}^{2+}]_{i}$ in DRG neurons to compare $\text{Ca}^{2+}$ amplitudes. Consistent with previous studies (Hong et al., 2000), I observed a mid-amplitude rise in $[\text{Ca}^{2+}]_{i}$ following netrin-1 addition (data not shown). This mid-amplitude response was found to occur during the same time period (10-12 minutes $\pm$ 1 minute) in which the marked rise in $[\text{Ca}^{2+}]_{i}$ following MotC addition was detected, thus confirming the rise induced by MotC was indeed a high-amplitude increase (data not shown).
Figure 3.1. MotC addition causes a high-amplitude rise in $[Ca^{2+}]_i$ in chick DRG growth cones. (A) Fluorescence image (340 nm excitation) of Chick DRG growth cone loaded with Fura-2-AM at time = 0 minutes. White box indicates the region of interest (ROI) used to quantify fluorescence intensities on the growth cones. Scale bar represents 10 $\mu$m. (B,C) Same growth cone following MotC addition at (B) time = 12 minutes and (C) time = 20 minutes. (D) Average fura-2-derived ratio values normalized to represent relative changes in $[Ca^{2+}]_i$, as denoted by $R_n$. Time = 0 represents application of MotC. These traces represent averages from 15 growth cones per condition.
**MotC requires influx of extracellular Ca\textsuperscript{2+} to induce growth cone collapse**

Based on the observed high-amplitude [Ca\textsuperscript{2+}]\textsubscript{i} increase, I examined whether the extracellular environment was the contributing source of Ca\textsuperscript{2+}. Utilizing a growth cone collapse assay, pre-incubation of DRG cultures with EGTA alone did not significantly increase the collapse rate as compared to control growth cones (Fig. 3.2A,B,E). When DRGs were cultured in the presence of MotC alone, almost all growth cones scored were collapsed (Fig. 3.2C,E). In contrast, pre-incubation with EGTA attenuated the collapsing effect of MotC back to control levels (Fig. 3.2D,E). To address whether internal Ca\textsuperscript{2+} stores contributed to this collapse response, I measured [Ca\textsuperscript{2+}]\textsubscript{j} levels in cultures pre-incubated with EGTA and then treated with MotC. Under these conditions, no significant difference between experimental and control conditions could be observed (Fig. 3.2F), suggesting MotC-mediated intracellular increases in Ca\textsuperscript{2+} are largely derived from the extracellular environment.

This observation was further substantiated when I applied cobalt (Co\textsuperscript{2+}), a general inhibitor of plasmalemmal high voltage-activated and mechanosensitive Ca\textsuperscript{2+}-permeable channels, to DRG cultures (Fig. 3.3). When incubated with Co\textsuperscript{2+} alone, the growth cone collapse response was not significantly different from control cultures (Fig. 3.3A,B,E). However, in comparison to MotC-treated cultures, DRGs pre-incubated with Co\textsuperscript{2+} and then treated with MotC were observed to collapse at a rate closer to control levels, indicating a significant attenuation of the MotC-collapsing effect (Fig. 3.3C,D,E). Taken together, these results suggest the collapsing effect of MotC is largely mediated through Ca\textsuperscript{2+} influx from the extracellular environment through Ca\textsuperscript{2+} channels at the plasma membrane.
Figure 3.2. Absence of extracellular calcium inhibits MotC-induced growth cone collapse. (A) Control DRG growth cone cultured for 24 hours and labeled with Alexa 488 phalloidin. Scale bar represents 10 μm. (B) DRG growth cone cultured for 24 hours, followed by 1 hour in the presence of EGTA. (C) Growth cone cultured for 24 hours, followed by 1 hour application of MotC. (D) Growth cones cultured for 24 hours, followed by 1 hour application of both 1 mM EGTA and MotC. (E) Quantification of growth cone collapse expressed as percent collapsed. n = 30 growth cones scored per condition with experiment done in triplicate. Error bars = standard error of the mean. * p < 0.01 as compared to MotC alone treatment. (F) Rn values following MotC addition at time = 0 minutes in the presence or absence of EGTA. Traces represent averages from 15 growth cones per condition.
**Figure 3.3.** Inhibition of Ca$^{2+}$ influx via Co$^{2+}$ blocks MotC-induced growth cone collapse. (A) Control DRG growth cones cultured for 24 hours and then labeled with Alex 488 phalloidin. Scale bar represents 10 μm. (B) DRG growth cones cultured for 24 hours, followed by 1 hour in the presence of 50 μM Co$^{2+}$. (C) Growth cones cultured for 24 hours, followed by 1 hour application of MotC. (D) Growth cones cultured for 24 hours, followed by 1 hour application of both 50 μM Co$^{2+}$ and MotC. (E) Quantification of growth cone collapse expressed as percent collapsed: n = 30 growth cones scored per condition with experiment done in triplicate. Error bars = standard error of the mean. * p < 0.01 as compared to MotC alone.
MotC activates and requires calpain to elicit its collapsing effects

As a result of this observed rise in intracellular Ca$^{2+}$, I next examined potential downstream targets of Ca$^{2+}$ involved in MotC-mediated collapse. Since calpain has previously been identified as a negative regulator of outgrowth that is activated following the induction of high Ca$^{2+}$ transients (Robles et al., 2003), I hypothesized that calpain was a downstream target of MotC. To test this, I utilized the collapse assay in conjunction with the fluorescence activity reporter molecule, t-BOC, which is a cell-permeable marker containing a quenched fluorescent signal that is released following calpain-specific cleavage. In this experiment, I incubated dissociated DRG cultures with t-BOC to achieve baseline activity levels of calpain (Fig. 3.4A,E). Culturing in the presence of the calpain inhibitor PD150606 abolished calpain activity (Fig. 3.4B,E). PD150606 is an equipotent inhibitor of both μ- and m-calpain, with high specificity for calpains relative to other proteases (Wang et al., 1996). Furthermore, PD150606 non-competitively blocks calpain activity by binding to the Ca$^{2+}$-binding domain of calpain (Wang et al., 1996). When I treated DRGs with MotC, almost a two-fold increase in calpain activity was observed (Fig. 3.4C,E). In contrast, pre-incubation with PD150606 prior to MotC treatment completely abolished calpain activity (Fig. 3.4D,E). Similar results were observed with a second calpain inhibitor, ALLM (Fig. 3.4E). To confirm neurites were in fact present in cultures treated with PD150606, enhanced visualization of Fig. 3.4B,D were prepared (Fig. 3.4F,G)

I next examined whether calpain was necessary for MotC-mediated collapse. In comparison to control growth cones, the collapse rate of cultures treated with PD150606 alone did not differ (Fig. 3.4H,I,L). However, in comparison to the high collapse rate of
Figure 3.4. Calpain is activated following MotC application and inhibition of calpain attenuates MotC effectiveness. (A) Dissociated control DRGs cultured for 24 hours and then treated for 30 minutes in the presence of 5 μM t-BOC. Scale bar represents 40 μm for panels A-D,F,G. (B) Cultures incubated for 24 hours, followed by pre-incubation with 30 μM PD150606 for 20 minutes, and then applied 5 μM t-BOC for 30 minutes. (C) Cultures incubated for 24 hours and then co-applied 5 μM t-BOC and MotC for 30 minutes. (D) DRG cultures incubated for 24 hours, pre-treated with 30 μM PD150606 for 20 minutes, and then co-applied 5 μM t-BOC + MotC for 30 minutes. (E) Quantification of assay expressed as percent of fluorescence intensity relative to control. n = 20 growth cones scored per condition with experiment done in triplicate. (F) Enhanced image of (B) for visualization of neurite processes. (G) Enhanced image of (D) for visualization of neurite processes. (H) Phalloidin staining of control DRG growth cone cultured for 24 hours. Scale bar represents 10 μm for panels H-K. (I) DRG treated for 1 hour in the presence of 30 μM PD150606 following 24 hour incubation. (J) DRG applied MotC for 1 hour following 24 hour culture. (K) Growth cone cultured for 24 hours, pre-treated with 30 μM PD150606 for 20 minutes and then applied MotC for 1 hour. (L) Quantification of growth cone collapse as expressed as percent collapsed. n = 30 growth cones scored per condition with experiment done in triplicate. Error bars = standard error of the mean. * p < 0.01 as compared to Sema5B alone.
those treated with MotC, addition of both PD150606 and MotC resulted in a significant attenuation of growth cone collapse (Fig. 3.4J,K,L). A range of PD150606 concentrations were tried and found that collapse could not be completely reduced, even at concentrations significantly higher than 30 μM (data not shown). When I repeated these growth cone collapse assays using ALLM, I found significant attenuation of MotC-mediated collapse as well, although not as robust as that observed with PD150606. I therefore conclude calpain is both activated and necessary to mediate the collapsing effects of MotC. However, inhibition of calpain was not sufficient to completely abolish the collapse response of MotC (Fig. 3.4J), suggesting calpain plays a partial role in mediating growth cone collapse.

MotC does not require calcineurin to induce growth cone collapse

It has previously been shown that the Ca^{2+}-dependent phosphatase calcineurin acts as a negative downstream effector molecule of outgrowth following induction of low Ca^{2+} transients (Lautermilch and Spitzer, 2000). To determine whether calcineurin activity contributes to the collapsing effects of MotC, I utilized the calcineurin inhibitors, Cyclosporin A (CsA) and deltamethrin (DM). CsA binds to cyclophilin A and competitively inhibits calcineurin at the same binding site as FKBP-FK506, an immunophilin-immunosuppressant complex (Liu et al., 1991). To ensure inhibition of calcineurin was specific and did not modulate other signaling pathways, we also used DM, a synthetic pyrethroid that directly inhibits calcineurin non-competitively (Enan and Matsumura, 1992). Tested against a host of other phosphatases, DM was shown to be
Figure 3.5. Inhibition of calcineurin does not attenuate MotC-mediated collapse. (A) Control DRG growth cone cultured for 24 hours. Scale bar represents 10 μm. (B) DRG growth cone cultured for 24 hours, followed by the addition of 50 nM CsA for 1 hour. (C) Growth cone cultured for 24 hours, followed by the addition of MotC for 1 hour. (D) Growth cone cultured for 24 hours, followed by pre-treatment of 50 nM CsA for 20 minutes, and then MotC for 1 hour. (E) Quantification of growth cone collapse expressed as percent collapsed. n = 30 growth cones scored per condition with experiment done in triplicate. Error bars = standard error of the mean.
specific against calcineurin (Enan and Matsumura, 1992). When DRGs were cultured in
the presence of CsA using the maximal effective concentration as determined by dose
response experiments (data not shown), no difference in growth cone collapse was
observed when compared to control cultures (Fig. 3.5A,B,E). Furthermore, when I
compared MotC-treated cultures in the presence and absence of CsA, those treated with
CsA did not show a significant difference compared to those treated without CsA (Fig.
3.5C,D,E). Similar results were also found when an alternate calcineurin inhibitor,
deltamethrin (DM), was used (Fig. 3.5E). These results show that calcineurin activity is
not involved in MotC-mediated growth cone collapse and supports the hypothesis that a
high-amplitude Ca²⁺ signal leads to calpain activation.

Calpain and Rho-ROCK signaling cascades are concurrently required
during MotC-mediated growth cone collapse

Since I found calpain to only be partially responsible for the collapsing effect of
MotC, combined with my previous observation that the Rho-ROCK pathway also
induced partial attenuation of the inhibitory effects of this molecule (To et al., 2006), I
hypothesized both pathways were required by MotC to induce maximal growth cone
collapsing effects. When cultures were treated with PD150606 and the ROCK inhibitor,
Y27632, the collapse rate did not differ significantly from control cultures receiving no
pharmacological blockers (Fig. 3.6A,B,E). In contrast, pre-incubation with both blockers
prior to MotC addition significantly attenuated the collapse response of MotC to a rate
similar to control levels (Fig. 3.6C,D,E). Based on these observations, I find that the
inhibition of both pathways has additive effects, suggesting both calpain- and Rho-
Figure 3.6. Inhibition of both calpain and ROCK attenuates the collapsing effect of MotC in an additive fashion. (A) Control DRG growth cone cultured for 24 hours. Scale bar represents 10 μm. (B) DRG growth cone cultured for 24 hours and then incubated with 30 μM PD150606 and 30 μM Y27632. (C) Growth cone cultured for 24 hours and then incubated with Sema5B for 1 hour. (D) Growth cone cultured for 24 hour, pre-incubated with 30 μM PD150606 + 30 μM Y27632 for 20 minutes, and then applied MotC for 1 hour. (E) Quantification of growth cone collapse expressed as percent collapsed. n = 30 growth cones scored per condition with experiment done in triplicate. Error bars = standard error of the mean. * p < 0.01 as compared to Sema5B alone. (F) Fluorescence image (340 nm excitation) of a growth cone loaded with Fura-2-AM and pre-treated with 30 μM Y27632 for 20 minutes, just prior to MotC addition. White box indicates the region of interest (ROI) used to quantify fluorescence intensity. Scale bar represents 10 μm. (G) Same growth cone following MotC addition at time = 30 minutes. (H) R_n values of neurons pre-treated with Y27632, followed by MotC addition. Also shown are R_n values recorded in parallel experiments in the presence of Y27632 but in the absence of MotC. White box indicates the ROI used to quantify fluorescence intensities within the growth cone. Scale bar represents 10 μm. Traces represent averages from 15 growth cones per condition.
Ctrl + PD 150606 + Y27632
MotC + PD 150606 + Y27632

E 100%

F 0' MotC + Y27632
G 30' MotC + Y27632

H 3

MotC + Y27632
Ctrl + Y27632

Time (minutes)
ROCK-mediated pathways act in combination to mediate the collapsing effects of MotC.

I next examined whether Ca\(^{2+}\) acts upstream of Rho-ROCK signaling by measuring Ca\(^{2+}\) levels in the presence of the ROCK inhibitor, Y27632 (Fig. 3.6F-H). While pre-incubation with Y27632 was able to block the collapsing effects of MotC as previously described (Fig. 3.6F,G), I find that there was little effect on the increased Ca\(^{2+}\) signal in response to MotC application (Fig. 3.6H). Furthermore, the EGTA experiments from Figure 2 show that chelation of extracellular Ca\(^{2+}\) was able to abolish the inhibitory effects of MotC (Figure 2E, EGTA alone compared to EGTA+MotC treatment). Taken together, this suggests MotC induces Ca\(^{2+}\) influx acts upstream of both Rho-ROCK and calpain-mediated pathways.
3.4 Discussion

In this study, I have used the synthetic growth cone collapsing molecule MotC to investigate pathways involved in growth cone collapse. I show that stimulation of extracellular Ca\(^{2+}\) influx through plasma membrane Ca\(^{2+}\) channels can induce a rapid, high-amplitude rise in \([\text{Ca}^{2+}]_i\), which in turn leads to growth cone collapse. This Ca\(^{2+}\) increase specifically activates calpain and not calcineurin. In addition, I provide novel evidence that calpain and Rho-ROCK signaling pathways are involved in combination during MotC-mediated collapse.

Previous studies have shown that depending on the stimulus regulating growth cone motility, the source of Ca\(^{2+}\) contributing to the \([\text{Ca}^{2+}]_i\) increase can vary (Henley et al., 2004; Hong et al., 2000). For example, netrin-1-induced \([\text{Ca}^{2+}]_i\) increase has been shown to result from both influx of extracellular Ca\(^{2+}\) and Ca\(^{2+}\) release from internal stores (Hong et al., 2000; Wang and Poo, 2005). In contrast, MAG-mediated rise in \([\text{Ca}^{2+}]_i\) was observed to be due to release of Ca\(^{2+}\) from internal stores only (Hong et al., 2000). Moreover, the rise in amplitude of \([\text{Ca}^{2+}]_i\) can markedly influence the downstream signals targeted (Gomez and Zheng, 2006; Kater and Mills, 1991; Lautermilch and Spitzer, 2000; Robles et al., 2003). In this study, I find MotC to induce a high-amplitude rise in \([\text{Ca}^{2+}]_i\) in comparison to the mid-amplitude rise in \([\text{Ca}^{2+}]_i\) stimulated by the chemoattractant netrin-1. This increase in \([\text{Ca}^{2+}]_i\) appears to be largely due to influx from the extracellular environment. Although it is known that transient receptor potential, mechanosensitive stretch-activated, and L- and N-type high voltage Ca\(^{2+}\)-channels are all present in growth cones and are blocked by Co\(^{2+}\) (Jacques-Fricke et al., 2006; Lipscombe
et al., 1988; Wang and Poo, 2005), future experiments will be required to selectively determine which channel(s) are involved in MotC-induced Ca\(^{2+}\)-influx.

Based on the high-amplitude [Ca\(^{2+}\)]\(_i\) rise in growth cones, I hypothesized that calpain activity may be involved in collapse. I found that MotC upregulates calpain activity and that pharmacological blockade of calpain abolished the collapsing effects of MotC. Although the downstream targets of calpain activation during growth cone collapse induced by an extracellular cue remain undefined, one potential target affecting motility may be calpain’s involvement in focal contact disassembly. Robles et al. (2003) showed that calpain activation via direct elevation of Ca\(^{2+}\) transients resulted in inhibition of Src activity, a tyrosine kinase involved in promoting the coupling of the actin cytoskeleton with adhesion receptors (Suter and Forscher, 2001). Other studies have also shown that active calpain cleaves talin-1, a cytoskeletal protein associated with adhesion complexes (Calle et al., 2006; Franco et al., 2004). Cleavage of talin-1 results in a disassociation between integrins and the actin cytoskeleton, leading to adhesion disassembly (Franco et al., 2004). Therefore, it is possible that activated calpain during growth cone collapse regulates either talin-1 or Src activity, and thus focal contact assembly. However, substrate adhesion is likely only one component necessary for growth cone collapse. Another critical step involved during collapse is the contractility of the actin cytoskeleton from the growth cone periphery. One effector molecule often associated with this step is the GTPase Rho.

Previous studies have shown that activated Rho induces growth cone collapse, due in part to its effects on actin contractility (Dent and Gertler, 2003; Meyer and Feldman, 2002; Ridley, 2001). I have previously shown that MotC induces growth cone collapse.
collapse in part due to activation of the Rho-ROCK pathway (To et al., 2006). Addition of MotC induces an upregulation of active Rho-GTP expression, and inhibition of either Rho or the downstream target ROCK partially abolishes the collapsing effects of MotC. Since inhibition of either calpain- or Rho-ROCK-mediated pathways result in incomplete attenuation of MotC-induced collapse, I examined whether both pathways may be concurrently activated. I found that inhibition of both Rho-ROCK and calpain-mediated pathways have additive effects, suggesting they are simultaneously involved during a MotC-mediated growth cone collapsing event. Furthermore, since inhibiting either pathway alone is not sufficient for rescuing all neurons from collapse, it is likely a threshold of activity is required for activation of either pathway and that some neurons, due to inherent differences, are predisposed towards collapse through activation of one pathway over the other. Future experiments will be necessary to clarify this possibility.

Interestingly, Rho has previously been shown to be a substrate for calpain proteolysis. Kulkarni et al. (2002) showed that in aortic endothelial cell cultures, calpain cleaves and inactivates RhoA during integrin signaling. Previously, I have examined both activated and total Rho protein levels following MotC addition (To et al., 2006). In this experiment, proteolytically processed fragments were not observed, indicating such an interaction unlikely between calpain and Rho in this neuronal system. While this study provides data placing Rho-ROCK signaling downstream of Ca\textsuperscript{2+}, future experiments will be required to directly measure Rho-ROCK activity following manipulation of [Ca\textsuperscript{2+}]\textsubscript{i} levels. One potential model could thus be that calpain induces uncoupling of focal contacts with the actin cytoskeleton leading to de-adhesion from the extracellular matrix, coupled to Rho-ROCK-induced retraction of the actin cytoskeleton from the periphery of
the growth cone, ultimately leading to growth cone collapse (Fig. 3.7). While each pathway could independently lead to growth cone collapse, my results in which inhibition of both pathways produces additive effects suggest that stimulation of multiple pathways is most likely involved. This model supports previous observations that semaphorins may also stimulate multiple pathways during growth cone collapse (K.C.W. To, unpublished). However, the possibility that cross-talk between Rho-ROCK and calpain signaling still can not be ruled out.

In this study I have utilized the inhibitory effects of MotC to elucidate signaling pathways involved in growth cone collapse. To the best of my knowledge, this is the first study to show concurrent activation of calpain- and Rho-ROCK signaling to mediate growth cone collapse induced by an inhibitory cue. Further analysis will be required to determine whether these pathways are required as a general phenomenon of collapse, or whether it is stimulus dependent. However, I provide evidence that growth cone collapse activates multiple pathways, each target likely impinging on a distinct mechanism required for the process of growth cone collapse.
Figure 3.7. Proposed model of multiple pathways modulated by MotC to induce growth cone collapse. MotC activates both calpain- and Rho-ROCK-mediated pathways to modulate collapse. Calpain signaling may impinge its effects on adhesion complexes, which in turn could uncouple from the actin cytoskeleton leading to de-adhesion from the substrate, while activated Rho-ROCK signaling may promote retraction of actin filaments. As a result, these two events most likely act in a coordinated manner to achieve growth cone collapse.
MotC

Ca^{2+}

Calpain

Rho-GTP

ROCK

Adhesion

Cytoskeleton

Growth cone collapse
3.5 References


4. COMBINED ACTIVATION OF CALPAIN AND CALCINEURIN DURING LIGAND-INDUCED GROWTH CONE COLLAPSE

4.1 Introduction

Axon guidance cues play a significant role in providing instructional guidance information to the growing axon's motile tip, the growth cone. The semaphorin family of secreted and membrane-associated guidance proteins influences motility in a large number of different types of neurons. Semaphorins can play a role in axon outgrowth as well as guidance, depending on the manner in which the growth cone of the extending axon encounters the cue (Henley and Poo, 2004; Pasterkamp and Verhaagen, 2006). If a spatial gradient of the guidance cue is presented, the growth cone will either turn towards or away from the source accordingly (Henley et al., 2004; Hong et al., 2000; Wong et al., 2002), whereas if a uniform signal is provided without any spatial reference, the growth cone will either extend or collapse accordingly (Mattson et al., 1988; McCobb et al., 1988). Previously, a novel vertebrate transmembrane Semaphorin was identified as Semaphorin 5B (Sema5B) (Adams et al., 1996). It has recently been characterized as an inhibitory Semaphorin, inducing inhibitory responses and growth cone collapse in a number of neuronal types, including chick dorsal root ganglion (DRG), sympathetic, and

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retinal ganglion cell neurons, as well as mouse cortical neurons (T.P. O'Connor, unpublished). However, the intracellular mechanisms that underlie these effects are unknown. Given the widespread expression of Sema5B in the developing embryo, and its function as a critical guidance cue for cultured neurons, we have examined the intracellular pathways that modulate the collapse of growth cones by Sema5B.

One intracellular messenger shown to play a central role in growth cone collapse is calcium. Previous studies have shown an optimal range of [Ca\(^{2+}\)]\(_i\) exists within the growth cone to promote neurite outgrowth (Kater and Mills, 1991; Lankford and Letourneau, 1991). Concentrations above or below this range promote growth cone collapse and retraction (Henley et al., 2004; Wen et al., 2004; Zheng, 2000). Furthermore, recent evidence has shown that different guidance cues can produce differences in the amplitude of the Ca\(^{2+}\) signal. For example, attractive netrin-1 has been shown to induce a mid-amplitude [Ca\(^{2+}\)]\(_i\) response (Hong et al., 2000), whereas a low-amplitude [Ca\(^{2+}\)]\(_i\) response was triggered in the presence of the inhibitory cue myelin-associated glycoprotein (MAG) (Henley et al., 2004). Of particular interest is how these different changes in [Ca\(^{2+}\)]\(_i\) affect downstream signaling events.

Of the many effectors of Ca\(^{2+}\) signaling, the phosphatase calcineurin and the protease calpain have been shown to be important for outgrowth and guidance of extending axons. In a study by Lautermilch and Spitzer (2000), it was shown that inhibition of calcineurin was necessary for neurite outgrowth in *Xenopus* spinal neurons. Furthermore, Wen et al. (2004) demonstrated a low-amplitude [Ca\(^{2+}\)]\(_i\) response could activate calcineurin. On the contrary, repulsive turning in *Xenopus* spinal neurons induced by large local [Ca\(^{2+}\)]\(_i\) transients requires activation of calpain. Based on these
results, Gomez and Zheng (2006) have recently proposed that a low-amplitude $[\text{Ca}^{2+}]_i$ rise results in calcineurin activation, while a high-amplitude $[\text{Ca}^{2+}]_i$ rise results in calpain activation. However, it remains unknown which guidance cues modulate $[\text{Ca}^{2+}]_i$ in this model and to what extent. Furthermore, it is unknown whether individual guidance cues may elicit responses involving multiple $\text{Ca}^{2+}$-mediated downstream pathways governing guidance and outgrowth, and whether cross-talk between such pathways occurs.

In the present study, we demonstrate that Sema5B induces growth cone collapse in chick DRGs via the influx of extracellular $\text{Ca}^{2+}$. The subsequent rise in $[\text{Ca}^{2+}]_i$ is characterized by an initial low-amplitude $[\text{Ca}^{2+}]_i$ increase, followed by a secondary high-amplitude $[\text{Ca}^{2+}]_i$ rise. Based on this distinct pattern of $[\text{Ca}^{2+}]_i$ change, combined with previous studies implicating calcineurin activation with low-amplitude rises in $[\text{Ca}^{2+}]_i$ and calpain activation with high-amplitude rises, we demonstrate for the first time using Sema5B as a model inhibitory guidance cue that both calcineurin- and calpain-mediated signaling cascades are required during a growth cone collapsing event. Furthermore, we provide novel evidence that following the addition of an inhibitory cue, cross-talk between these pathways via calpain-mediated cleavage of calcineurin occurs.
4.2 Materials and Methods

**Inhibitors and antibodies.** A truncated secreted form of Sema5B containing only the Semaphorin domain in pDisplay vector (Invitrogen) was stably transfected into HEK293 cells. Total supernatant from this stably expressing cell line was collected and Sema5B concentrated to approximately 222 nM. Equivalent amounts of control supernatant were also collected from HEK293 cells that were transfected with the empty pDisplay vector. Stock Fura-2-AM (Invitrogen) was made to 1 mg/ml in chloroform, aliquoted, placed in a vacuum evaporator to boil off chloroform, and then stored at -80°C. EGTA (Fisher Scientific) was prepared fresh in DMEM (Sigma Aldrich) at a working concentration 1 mM. CoCl₂ (Fisher Scientific) was prepared fresh in DMEM at a working concentration of 50 μM. Cyclosporin A (CsA) and Deltamethrin (DM) were obtained from Calbiochem and prepared as previously described (Lautermilch and Spitzer, 2000). Briefly, CsA and DM were made fresh for each experiment by dissolving in a 1:1 mixture of ethanol and DMSO. Stock concentrations of CsA and DM were 50 μM and 5 μM, respectively, while working concentrations of 50 nM CsA and 5 nM DM were diluted in DMSO. PD150606 and ALLM were obtained from Calbiochem. Both inhibitors were prepared by dissolving in DMSO at stock concentrations of 30 mM and 10 mM, respectively. Working concentrations of 30 μM PD150606 and 10 μM ALLM were diluted in DMSO. Anti-calcineurin A antibody (Stressgen Bioreagents Corp.) was used at a concentration of 0.4 μg/ml. 7-amino-4-chloromethylcoumarin, t-BOC-L-leucyl-L-methionine amide (t-BOC, Invitrogen) was made to a stock concentration of 10 mM in DMSO and used at a working concentration of 5 μM dissolved in DMEM.
Preparation of cell cultures. 18 mm glass coverslips (Fisher Scientific) in 12-well culture dishes (Falcon) were treated overnight at 37°C with 1 μg/ml laminin (Chemicon) and 100 μg/ml poly-L-lysine (Sigma Aldrich). Lumbar explants of embryonic day 10 (E10) White Leghorn chick DRGs were cultured in these wells the next day with 50 ng/ml NGF (Invitrogen)-supplemented DMEM containing phenol red (Sigma Aldrich) for 24 hours at 37°C.

Sema5B growth cone collapse assays. Sema5B was added to cultures for 1 hour at 37°C. Explants were then fixed with 3.7% formaldehyde solution in PBS for 15 minutes at room temperature. After fixation, cultures were washed in PBS (2 x 5 minutes), followed by 5 minutes in PBS containing 0.1% Triton X-100, and then by PBS (2 x 5 minutes) once more. To visualize growth cone morphology, neurons were stained with 2 units of Alexa Fluor 488 phalloidin (Invitrogen) diluted into 200 μl PBS containing 1% BSA for 30 minutes.

Calcium imaging. Measurements of intracellular free calcium concentration ([Ca^{2+}]_i) were performed using the dual-excitation ratio method, employing an imaging system (Northern Eclipse, Empix Imaging Inc.) in conjunction with a Zeiss Axioskop FS Plus microscope fitted with an Achroplan 100x water immersion objective (Carl Zeiss Canada). Dissociated cultures were used to allow for greater uptake of the Ca^{2+} reporter molecule, fura-2-AM (Invitrogen). Isolated ganglia were enzymatically and mechanically dissociated using 0.1% trypsin (Stem Cell Technologies) as previously described (Nakamoto and Shiga, 1998), and then cultured in DMEM containing phenol red (Sigma Aldrich) supplemented with 50 ng/ml NGF (Invitrogen) for 24 hours at 37°C. Details of the methods employed have been previously presented (Church et al., 1998). In brief,
DRGs were incubated with 5 μM fura-2-AM in the presence of 0.02% Pluronic F-127 for 45 minutes at 37 °C and then placed in DMEM without phenol red for 15 minutes to ensure de-esterification of the fluorophore. Subsequently, coverslips were mounted in a temperature-controlled recording chamber (Warner Instruments) at 37°C under static conditions. Fluorescence emissions measured at 510 ± 20 nm from neurons loaded with fura-2 were detected by a cooled CCD camera (Retiga EXi, QImaging, Surrey, BC, Canada) and collected from regions of interest placed on individual growth cones. Ratio pairs were acquired at 10 sec intervals throughout the course of an experiment and analyzed off-line. Raw emission intensity data at each excitation wavelength (340 and 380 nm) were corrected for background fluorescence prior to calculation of a ratio. To highlight relative changes in [Ca\(^{2+}\)], ratio values were normalized by dividing the change in fura-2-derived ratio values evoked by a given experimental maneuver by the ratio value just prior to addition of stimulus, and averaged from 15 growth cones per condition (expressed as R\(_n\)). For experiments using EGTA to chelate extracellular Ca\(^{2+}\), [Mg\(^{2+}\)] was increased to 1.8 mM, and 1 mM EGTA was added.

**Preparation of cell extracts.** Mouse cortical neurons were isolated and dissociated from E12 mouse embryos as previously described (Alifragis et al., 2004). Cortical neurons were then cultured in Neurobasal medium (Gibco) containing 10% FBS (Colorado Serum Company), 2 mM glutamine (Fisher Scientific), and a 1:50 dilution of B27 (Invitrogen) for 24 hours, followed by 2 washes in Neurobasal medium containing 2 mM glutamine, 1:50 B27, and 25 μM glutamate to stimulate neurite outgrowth, and then allowed to incubate for another 48 hours at 37°C. Following this incubation, experimental cultures were pre-incubated in the presence of PD150606 for 20 minutes, while control cultures
received equivalent DMSO treatments. Cultures were then incubated with Sema5B or control media for 60 minutes, at which point all cultures were lysed and prepared for Western blot analysis as previously described (Koh et al., 2006). Briefly, equal protein loading levels were determined using a colorimetric protein assay kit (BioRad), and equal amounts of protein were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a nitrocellulose membrane (Amersham Biosciences). Membranes were then washed with TBS containing 0.1% Tween 20 (TBS-T), followed by 1 hour incubation in blocking solution containing 3% dry milk in TBS-T at room temperature, and then incubated overnight in anti-calcineurin A antibody 1:2000 overnight at 4°C. Afterwards, membranes were washed in TBS-T (3x5 minutes), incubated in horseradish peroxidase-conjugated donkey anti-rabbit (Jackson Laboratories) 1:3000 for 1 hour at room temperature and then visualized using ECL-based detection reagents (Amersham Biosciences). Equal protein loading levels were also confirmed by immunoblotting anti-β-tubulin (Sigma).

**t-BOC calpain activity assay.** To allow for more uniform uptake of the fluorescence calpain activity reporter molecule, dissociated cultures were used. Calpain activity was measured for the following conditions: control cultures, cultures pre-incubated with 30 μM PD150606 for 20 minutes, cultures incubated with Sema5B for 10 or 30 minutes, and cultures pre-incubated with 30 μM PD150606 for 20 minutes followed by Sema5B addition for 10 or 30 minutes. t-BOC was added 10 minutes prior to visualization of calpain activity in all experimental conditions. Fluorescent imaging of calpain activation was determined under live cell conditions.
Quantification. Growth cones were scored as collapsed according to Fan et al. (1993). Specifically, growth cones lacking lamellipodia and extending 4 or less filopodia were scored as collapsed. Each experiment was done in triplicate with 30 growth cones scored per condition. Calpain activation using the fluorescent t-BOC reporter molecule was quantified by measuring average fluorescent signal intensities for 20 growth cones per condition and dividing by baseline control intensity. Microsoft Excel was used to calculate the standard error of mean and standard deviation for each experimental condition. Statistical significance of differences between conditions was determined by ANOVA and Student's t-test using SPSS statistical software version 11.0 for Windows.
4.3 Results

**Sema5B causes a rise in \([\text{Ca}^{2+}]_i\) during growth cone collapse**

Since \(\text{Ca}^{2+}\) has been shown to play a central role as a mediator of growth cone motility in numerous cell types and conditions (Bolsover, 2005; Gomez and Zheng, 2006; Henley and Poo, 2004), we sought to examine whether \(\text{Ca}^{2+}\) also plays a role in Sema5B signaling by measuring the effects of Sema5B on \([\text{Ca}^{2+}]_i\) in the growth cones of dissociated E10 chick DRGs loaded with Fura-2 (Fig. 4.1). As illustrated in Fig. 4.1C, the addition of Sema5B resulted in an initial low-amplitude rise in \([\text{Ca}^{2+}]_i\), which commenced 7 minutes (± 1.5 minutes; \(n = 15\) growth cones per condition) after the addition of the guidance cue and persisted for approximately 9 minutes (± 1.9 minutes) before giving way to a marked rise in \([\text{Ca}^{2+}]_i\) that continued for the duration of the experiment. These rises in \([\text{Ca}^{2+}]_i\) were concomitant with growth cone collapse. Specifically, observable morphological changes typically occurred 5-10 minutes following Sema5B addition, where filopodia began retracting back towards the growth cone. At 20-40 minutes following Sema5B addition, complete collapse was observed. Neither a rise in \([\text{Ca}^{2+}]_i\) nor growth cone collapse were detected in parallel experiments in which supernatant isolated from HEK293 cells mock-transfected with the pDisplay vector alone was applied (Fig. 4.1C). Since it has been previously postulated that high- and low-amplitude rises in \([\text{Ca}^{2+}]_i\) result in chemorepulsion while mid-amplitude rises result in chemotraction (Gomez and Zheng, 2006; Kater and Mills, 1991), for comparative purposes we measured \([\text{Ca}^{2+}]_i\) in Fura-2-loaded DRG growth cones treated with the chemoattractant
**Figure 4.1.** Addition of Sema5B induces a significant rise in $[\text{Ca}^{2+}]_{i}$ in chick DRG growth cones.

(A) Fluorescence image (340 nm excitation) of extending chick DRG growth cone loaded with Fura-2-AM just prior to Sema5B addition. White box indicates the region of interest (ROI) used to quantify fluorescence intensities on the growth cones. Scale bar represents 10 μm. (B,C) Same growth cone at (B) 8 and (C) 25 minutes following Sema5B addition. (D) Normalized changes in fura-2-derived ratio values highlighting relative changes in $[\text{Ca}^{2+}]_{i}$, expressed as $R_{n}$, evoked by the addition of Sema5B at time = 0. Also shown are $R_{n}$ values observed in parallel experiments in the absence of Sema5B. The records represent averages from 15 growth cones per condition.
netrin-1 (Fig. 4.2). A mid-amplitude increase in \([\text{Ca}^{2+}]\), occurred following netrin-1 addition, confirming that Sema5B induced relatively low- and high-amplitude rises in \([\text{Ca}^{2+}]\), as illustrated in Fig. 4.1C.

**Sema5B-induced increases in \([\text{Ca}^{2+}]\) reflect \(\text{Ca}^{2+}\) influx**

We next examined potential sources for the rise in \([\text{Ca}^{2+}]\). Depending on the guidance cue, \(\text{Ca}^{2+}\) sources can include both the extracellular environment and intracellular stores (Henley et al., 2004; Hong et al., 2000). For example, attractive netrin-1 induces \(\text{Ca}^{2+}\) influx through plasma membrane \(\text{Ca}^{2+}\) channels and \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release from internal stores (Hong et al., 2000). On the contrary, MAG-mediated repulsion is reported to be due to release of \(\text{Ca}^{2+}\) from internal stores alone (Henley et al., 2004). To examine whether Sema5B-induced increases in \([\text{Ca}^{2+}]\) were consequent upon \(\text{Ca}^{2+}\) influx, we performed growth cone collapse assays in the presence of 1 mM EGTA to chelate \(\text{Ca}^{2+}\) from the extracellular environment (Fig. 4.3). In the presence of the \(\text{Ca}^{2+}\) chelator alone, little collapse was observed and growth cones appeared similar to control growth cones (Fig. 4.3A,B,E). In parallel experiments, Sema5B addition alone to cultures resulted in almost all growth cones collapsing (Fig. 4.3C,E). However, when DRG explants were cultured in the presence of both Sema5B and EGTA, the collapsing effects of Sema5B were significantly attenuated back toward basal levels (Fig. 4.3D,E). Furthermore, when applied in the presence of EGTA, Sema5B failed to induce marked increases in \([\text{Ca}^{2+}]\) (Fig. 4.3F), suggesting that Sema5B was acting to promote \(\text{Ca}^{2+}\)
**Figure 4.2.** Addition of netrin-1 induces a mid-amplitude rise in $[\text{Ca}^{2+}]$. (A)

Fluorescence image (340 nm excitation) of Fura-2-AM loaded growth cone just prior to netrin-1 addition. ROI indicated by white box. Scale bar represents 10 μm. (B) Same growth cone 30 minutes following netrin-1 application. (C) Quantification of Rn values induced by netrin-1 addition over time. Also shown are Rn values observed in parallel control experiments in the absence of netrin-1. Traces represent averages from 15 growth cones per condition.
Fig. S1. Addition of netrin-1 induces a mid-amplitude rise in [Ca\textsuperscript{2+}]. (A) Fluorescence image (340 nm excitation) of Fura-2-AM loaded growth cone just prior to Netrin-1 addition. ROI indicated by white box. Scale bar represents 10 μm. (B) Same growth cone 30 minutes following Netrin-1 application. (C) Quantification of Rn values induced by Netrin-1 addition over time. Also shown are Rn values observed in parallel control experiments in the absence of Netrin-1. Traces represent averages from 15 growth cones per condition.
**Figure 4.3.** Sema5B-induced growth cone collapse is markedly attenuated in the presence of the external Ca\(^{2+}\)-chelator, EGTA.

(A) DRG growth cones cultured under control conditions for 24 hours. Scale bar, 10 μm.  
(B, C, D) DRG growth cones cultured for 24 hours, followed by 1 hour in the presence of (B) 1 mM EGTA, (C) Sema5B, or (D) 1 mM EGTA and Sema5B.  
(E) Quantification of growth cone collapse under the conditions indicated on the Figure, expressed as percent collapsed. n = 30 growth cones scored per condition with experiments done in triplicate.  
Error bars = standard error of the mean. * p < 0.01 compared to Sema5B alone.  
(F) R\(_n\) values (representing relative changes in \([\text{Ca}^{2+}]_i\)) during EGTA-containing media in parallel experiments conducted in the presence or absence of Sema5B. Traces represent averages from 15 growth cones per condition.
A Ctrl

B Ctrl + EGTA

C Sema

D Sema5B + EGTA

E

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%

Percent Collapsed (%)

EGTA - - + +
Sema5B - - + +

F

Time (minutes)
influx. In support of this possibility, growth cone collapse induced by Sema5B was markedly attenuated by cobalt (Co²⁺), a broad-spectrum blocker of the plasmalemmal high voltage-activated and mechanosensitive Ca²⁺-permeable channels known to be present in growth cones (Fig. 4.4C,D,E). Applied alone, Co²⁺ failed to significantly affect growth cone collapse (Fig. 4.4A,B,E). These results suggest Sema5B induces growth cone collapse in DRGs in large part via Ca²⁺ influx from the extracellular environment.

**Calpain and calcineurin are involved in Sema5B-mediated collapse**

We next examined what downstream Ca²⁺ targets were activated during the process of growth cone collapse. A previous study by Robles et al. (2003) showed that induction of localized high [Ca²⁺]ᵢ levels on one side of the growth cone via caged Ca²⁺ release resulted in an upregulation of calpain activity in this region of the growth cone. Furthermore, this activation in turn led to slowing of neurite outgrowth and repulsive turning (Robles et al., 2003). Based on the observed high-amplitude rise in [Ca²⁺]ᵢ following Sema5B addition, we hypothesized calpain activation may be a downstream target of this intracellular Ca²⁺ change. Using r-BOC, a fluorescence reporter molecule for calpain activity, we performed a time-course experiment to monitor calpain levels following Sema5B addition. We found calpain activity did not increase significantly above control levels in the first 10 minutes of Sema5B application, but did markedly rise by 30 minutes (Fig. 4.5A,C,E,G). Moreover, we found cultures incubated in the presence of the calpain inhibitor PD150606, which has a high affinity for the Ca²⁺ binding site of calpain, dramatically reduced calpain activity (Fig. 4.5B,D,F,G). Similar results were
Figure 4.4. Co^{2+}, a broad spectrum blocker of high voltage-activated Ca^{2+} channels, attenuates Sema5B-induced growth cone collapse.

(A) DRG growth cones cultured under control conditions for 24 hours. Scale bar, 10 µm. (B,C,D) DRG growth cones cultured for 24 hours, followed by 1 hour in the presence of (B) 50 µM Co^{2+}, (C) Sema5B, or (D) 50 µM Co^{2+} and Sema5B. (E) Quantification of growth cone collapse under the conditions shown on the Figure, expressed as percent collapsed. n = 30 growth cones scored per condition with experiments done in triplicate. Error bars = standard error of the mean. * p < 0.01 compared to Sema5B alone.
A Ctrl
B Ctrl + Co²⁺
C Sema5B
D Sema5B + Co²⁺

E 100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%

Percent Collapsed (%)

Co²⁺  Sema5B
-  -  +  +  +
Figure 4.5. Calpain activity is upregulated and necessary for Sema5B-mediated growth cone collapse.

(A,B) Calpain activity measured using the reporter molecule r-BOC for control dissociated DRG neurons following a 20 minute application of (A) DMEM only or (B) 30 μM PD150606. Scale bar represents 40 μm. (C,D) Calpain activity of neurons following (C) a 10 minute application of Sema5B or (D) a 20 minute pre-incubation with 30 μM PD150606 and then 10 minute addition of Sema5B. (E,F) Calpain activity of neurons following (E) a 30 minute application of Sema5B or (F) a 20 minute pre-incubation with 30 μM PD150606 and then 30 minute addition of Sema5B. (G) Quantification of calpain activity assay expressed as percent of fluorescence intensity relative to control. n = 20 growth cones scored per condition with experiments done in triplicate. (H) Control DRG growth cone. Scale bar represents 10 μm. (I) DRG growth cone following 1 hour in the presence of 30 μM PD150606. (J) Growth cone following 1 hour application of Sema5B. (K) Growth cone following 20 minute pre-incubation with 30 μM PD150606 and then 1 hour addition of Sema5B. (L) Quantification of growth cone collapse expressed as percent collapsed. n = 30 growth cones scored per condition with experiment done in triplicate. Error bars = standard error of the mean. * p < 0.01 as compared to Sema5B alone.
A  Ctrl  30'
B  Ctrl + PD150606  30'
C  Sema5B  10'
D  Sema5B + PD150606  10'
E  Sema5B + PD150606  30'
F  Sema5B + PD150606  30'
G  Capillar Activity (%)  180%
H  Ctrl  
I  Ctrl + PD150606
J  Sema5B
K  Sema5B + PD150606
L  Percent Collapsed (%)  100%
obtained with another cell-permeable calpain inhibitor, ALLM (results not shown).

To examine whether calpain activity is indeed necessary for Sema5B-mediated collapse, we utilized PD150606 in growth cone collapse assays (Fig. 4.5H-L). In the presence of PD150606 alone, growth cone collapse was not significantly different from that observed in control cultures (Fig. 4.5H,J,L). Following Sema5B addition, a high proportion of growth cones collapsed as expected (Fig. 4.5J,L). However, performing the same assay in the presence of both Sema5B and PD150606 alleviated the collapsing effects of Sema5B (Fig. 4.5K,L). Using ALLM provided similar results (data not shown). Interestingly, Sema5B-induced growth cone collapse was only partially reduced when calpain activity was blocked, suggesting the involvement of additional Ca^{2+}-dependent downstream targets.

Based on the incomplete attenuation of Sema5B-mediated collapse in the presence of PD150606, coupled to the fact that an initial low-amplitude rise in \([\text{Ca}^{2+}]_i\) was observed, we postulated a second intracellular Ca^{2+}-dependent pathway may be involved. Calcineurin has been shown to be activated following small rises in \([\text{Ca}^{2+}]_i\), resulting in the inhibition of neurite extension (Lautermilch and Spitzer, 2000; Wen et al., 2004). We therefore hypothesized that calcineurin might be a second target modulated by Sema5B-Ca^{2+} signaling (Fig. 4.6). Utilizing the same growth cone collapse assay, cultures incubated in the presence of the calcineurin inhibitor, Cyclosporin A (CsA), did not show a significant difference from control cultures when scoring for collapse (Fig. 4.6,B,E). However, when we compared the effects of Sema5B in the presence and absence of CsA, cultures receiving CsA exhibited a significant reduction in Sema5B-mediated collapse (Fig. 4.6C,D,E). Since CsA inhibits calcineurin indirectly by binding
**Figure 4.6.** Inhibition of calcineurin with CsA or DM significantly reduces the Sema5B-mediated collapse response.

(A) Control DRG growth cones cultured for 24 hours. Scale bar represents 10 μm. (B) DRG growth cones cultured for 24 hours, followed by 1 hour in the presence of 50 nM CsA. (C) Growth cones cultured for 24 hours, followed by 1 hour application of Sema5B. (D) Growth cones cultured for 24 hours, followed by 1 hour application of both 50 nM CsA and Sema5B. (E) Quantification of growth cone collapse expressed as percent collapsed. n = 30 growth cones scored per condition with experiments done in triplicate. Error bars = standard error of the mean. * p < 0.01 as compared to Sema5B alone.
to cyclophilin A (Lautermilch and Spitzer, 2000), we employed an alternate pharmacological agent, deltamethrin (DM), which directly binds to and inhibits calcineurin. Under these conditions, similar results were observed (Fig. 4.6E).

Calpain and calcineurin act in combination following Sema5B-mediated growth cone collapse

Based on the above findings, it appears inhibition of either calcineurin or calpain alone is insufficient to completely abolish the growth cone collapsing effects of Sema5B, suggesting both are potentially involved. To test this, we performed collapse assays in the presence of both CsA and PD150606 (Fig. 4.7). Comparing control cultures incubated in the presence of both CsA and PD150606 with cultures receiving no inhibitors, we found that CsA + PD150606-treated cultures had similar collapse rates as compared to non-treated cultures (Fig. 4.7A,B,E). In addition, CsA + PD150606-treated cultures incubated with Sema5B displayed significantly less collapse as compared to cultures incubated with Sema5B alone (Fig. 4.7C,D,E). Analysis of the collapse rate between experiments of Sema5B + CsA + PD150606 (~21%), Sema5B + CsA (~44%), and Sema5B + PD150606 (~35%) indicated that cultures incubated with both CsA and PD150606 display significantly lower rates of collapse as compared to those incubated with either inhibitor alone (p < 0.05, ANOVA). These results indicate a partial additive effect, suggesting Sema5B requires activation of both calpain and calcineurin to elicit maximal collapsing effects.
Figure 4.7. Inhibition of both calpain and calcineurin reduces growth cone response to Sema5B in an additive fashion.

(A) Control DRG growth cones cultured for 24 hours. Scale bar represents 10 µm. (B) DRG growth cones cultured for 24 hours, followed by 1 hour in the presence of 30 µM PD150606 and 50 nM CsA. (C) Growth cones cultured for 24 hours, followed by 1 hour application of Sema5B. (D) Growth cones cultured for 24 hours, followed by 1 hour application of 30 µM PD150606 + 50 nM CsA + Sema5B. (E) Quantification of growth cone collapse expressed as percent collapsed. n = 30 growth cones scored per condition with experiments done in triplicate. Error bars = standard error of the mean. * p < 0.01 as compared to Sema5B alone. (F) Lysates of mouse cortical neuronal cultures treated under various conditions probed for proteolytic fragments of calcineurin using a rabbit polyclonal antibody. In experimental conditions where only Sema5B is added, 60 kDa, 48 kDa, and 45 kDa fragments are present. In contrast, cultures incubated in both Sema5B and PD150606 yields only a 60 kDa band. (G) Quantification of calpain activity assay to examine a directional interaction between calpain and calcineurin, expressed as percent of t-BOC-derived fluorescence intensity relative to control. n = 20 growth cones scored per condition with experiment done in triplicate. Error bars = standard error of the mean. * p < 0.01 as compared to Sema5B alone.
A Ctrl  B Ctrl + CsA + PD150606
C Sema5B  D Sema5B + CsA + PD150606

E

Percent Collapsed (%)  100%  90%  80%  70%  60%  50%  40%  30%  20%  10%  0%

CsA  PD150606  Sema5B
-  +  -  +

F

60 kDa  48 kDa  45 kDa
Tubulin
PD150606  -  +  -  +
Sema5B  -  -  +  +

G

Gelatinase Activity (%)

0%  20%  40%  60%  80%  100%  120%  140%  160%  180%

CsA  Sema5B
-  +  -  +

142
We next examined a potential relationship between calpain and calcineurin as previous studies have indicated in other systems that calpain may regulate calcineurin activity by proteolytic cleavage of the full length calcineurin protein (Wu et al., 2004; Shioda et al., 2006). Previous results have also shown that three cleavage sites in calcineurin exist, producing 60 kDa, 48 kDa and 45 kDa forms, with the latter two smaller fragments being constitutively active (Uchino et al., 2003; Wu et al., 2004). Based on these previous studies, combined with the observation that both calpain and calcineurin contribute to Sema5B-mediated collapse, we hypothesized that calpain activation may induce cleavage of calcineurin, producing a truncated activated product. To test this hypothesis, we cultured dissociated mouse cortical neurons, incubated in the presence of PD150606, Sema5B, or both, and examined whether cleavage of calcineurin occurred (Fig. 4.7F). Neuron-enriched cultures of mouse cortical neurons were used in this case to avoid the inherent contamination of chick DRG cultures with non-neuronal cell types. Moreover, growth cones of mouse cortical neurons collapse following Sema5B addition (T.P. O’Connor, unpublished). In control cultures, low levels of endogenous proteolytic products were observed, while addition of PD150606 attenuated these fragments. Moreover, addition of Sema5B induced an increase in calcineurin proteolytic fragments, whereas cortical neurons receiving both Sema5B and PD150606 showed a reduction of the cleavage products (Fig. 4.7F). These data provide evidence that Sema5B-mediated calpain activation can result in the proteolytic cleavage of calcineurin. Furthermore, they provide evidence that calpain- and calcineurin-mediated collapse pathways are not necessarily independent of one another, and that interaction between these two pathways can occur. To address whether this interaction was unidirectional, we
performed a t-BOC calpain activity assay using CsA. While addition of Sema5B produced a significant upregulation of calpain activity as expected, in the presence of CsA and Sema5B, no significant difference in calpain activity could be detected as compared to Sema5B alone (Fig. 4.7G). This suggests calcineurin in this particular system does not regulate the activity of calpain, and that calpain-mediated cleavage of calcineurin is a unidirectional event. These results thus show that in the presence of Sema5B, calpain activation is sufficient to induce cleavage of calcineurin, with both calpain and calcineurin contributing to the growth cone collapsing effects of Sema5B.
4.4 Discussion

We demonstrate in this study that Sema5B induces growth cone collapse by inducing an influx of Ca²⁺ through Ca²⁺-sensitive Ca²⁺ channels, thereby causing a rise in [Ca²⁺]. Interestingly, this increase in [Ca²⁺], occurs in a distinct two-step profile; an initial low-amplitude [Ca²⁺]ₖ rise, followed by a subsequent high-amplitude rise in [Ca²⁺].

Furthermore, using Sema5B as a model inhibitory guidance cue, we demonstrate for the first time that calpain and calcineurin activity are both involved during a growth cone collapsing event. We also show in our system that upon growth cone collapse, not only can guidance cue-induced changes in [Ca²⁺]ₖ dictate downstream targets, but interaction amongst these effector molecules may occur.

In previous studies examining guidance cues and calcium, the inhibitory effects of MAG were shown to only require a Ca²⁺ release from intracellular stores (Henley et al., 2004), while Sema3A has been shown not to require Ca²⁺ at all to elicit its inhibitory effects (Shim et al., 2005; Song et al., 1998). In contrast, we show that in the presence of Sema5B, the source of Ca²⁺ contributing to the intracellular increase is largely from the extracellular environment. Thus, it appears that the specific role Ca²⁺ plays in modulating motility is guidance cue-specific. Importantly, although neurotransmitters can elicit Ca²⁺ influx through ionotrophic receptors, and thereby modulate growth cone activity, other guidance cues can open plasma membrane Ca²⁺ channels by a mechanism other than a change of membrane voltage (Bolsover, 2005). Although it remains unknown whether Sema5B induces membrane depolarization, L- and N-type high voltage-activated Ca²⁺ channels, as well as transient receptor potential (TRP) channels and mechanosensitive
stretch-activated channels, are present in growth cones (Jacques-Fricke et al., 2006; Lipscombe et al., 1988; Wang and Poo, 2005) and are all blocked by Co^{2+}. Experiments employing selective inhibitors will be required to determine precisely which of these pathway(s) mediate the effect of Sema5B to promote Ca^{2+} influx.

Following the identification of Ca^{2+} as an essential regulator of Sema5B collapse, we examined the downstream targets of this cue. Robles et al. (2003) previously showed that repulsive turning induced by local high Ca^{2+} transients in filopodia was calpain-dependent. We show in this study that calpain activity is not upregulated following 10 minute application of Sema5B, but does markedly rise following 30 minutes of Sema5B addition. This is in agreement with Robles et al. (2003) and consistent with the results from Fig. 1. Taken together, we find calpain activity is only upregulated during Sema5B-induced high-amplitude rises in [Ca^{2+}]. We also show with pharmacological inhibitors of calpain that this protease is indeed necessary for Sema5B-induced growth cone collapse.

Previously, it was shown that Ca^{2+}-mediated calpain activation regulates integrin-mediated adhesion through proteolytic processing of the cytoskeletal protein talin (Franco et al., 2004) and/or the tyrosine kinase Src (Robles et al., 2003). Since talin plays a key role in coupling the integrin-mediated cell adhesion to the actin cytoskeleton (Critchley, 2005; Franco et al., 2004), one potential downstream pathway of Sema5B-induced calpain activation could therefore be the local uncoupling of receptor-cytoskeletal linkages by calpain-dependent cleavage of talin. While the activation of calpain and potentially the disruption of adhesion complexes is certainly a plausible mechanism contributing to growth cone collapse, it is unlikely the only event since we have shown in this study that calpain inhibition only partially blocked Sema5B-mediated collapse. In
In this regard, it should be noted that PD150606 was used at the maximal effective concentration as determined from dose response experiments (data not shown). Strong evidence indicates that the actin cytoskeleton also plays a significant role during the process of growth cone collapse (Fan et al., 1993; Gallo, 2006; Jin and Strittmatter, 1997).

Calcineurin, a phosphatase previously implicated in Ca\(^{2+}\)-mediated growth cone collapse and shown to modulate the actin cytoskeleton (Dent and Meiri, 1998; Lautermilch and Spitzer, 2000), is a potential candidate as a second pathway activated via Sema5B. In this study, we find pharmacological blockade of calcineurin with two different inhibitors partially attenuates the effectiveness of Sema5B as an inhibitory cue. Again, dose response experiments were utilized to derive the maximum effective concentration of CsA (data not shown). Previous studies have shown activated calcineurin dephosphorylates and activates another phosphatase, protein phosphatase 1 (PP1) to induce repulsive growth cone turning (Lautermilch and Spitzer, 2000; MacKintosh and MacKintosh, 1994). In this light, it seems plausible PP1 may be a potential downstream target of calpain-activated calcineurin. An alternate downstream target of calcineurin could be dephosphorylation of the growth and plasticity associated protein GAP43 (Dent and Meiri, 1998; He et al., 1997; Lautermilch and Spitzer, 2000), which has previously been shown to promote actin polymerization (Dent and Meiri, 1998; He et al., 1997) and is concentrated in growth cones (Liu and Storm, 1989). Indeed, Lautermilch and Spitzer (2000) have shown that inducing [Ca\(^{2+}\)]\(_i\) transients lowers the amount of phosphorylated GAP43 and that inhibition of calcineurin returns GAP43 to baseline levels. One model therefore would be that Sema5B-mediated dephosphorylation
of GAP43 via calcineurin may result in capping of actin filaments and ultimately an inhibition in motility.

Given our results indicating the involvement of both calpain and calcineurin activity following Sema5B addition, we addressed whether simultaneous inhibition of both effector molecules yielded additive effects. Examining Sema5B-mediated collapse under these conditions, we found inhibition of both proteins were indeed additive. These results suggest that both pathways are also independently activated in response to Sema5B, as opposed to being downstream targets to one another. Furthermore, it should be noted that inhibiting either pathway alone is not sufficient for rescuing all neurons from collapse. This would suggest a threshold of activity of either pathway that can induce collapse or that some neurons are biased towards collapse through the activation of one pathway over the other. Further analysis will be necessary to distinguish between these two possibilities. Finally, one can not rule out the possibility that the pharmacological inhibitors used were not of maximal specificity and/or effectiveness. However, since we have used concentrations of inhibitors that produced maximal effects, combined with our use of multiple, analogous inhibitors in each experiment, we believe such possibilities have been mitigated.

To further examine the complexities of downstream Ca\(^{2+}\) signaling, we examined whether calpain and calcineurin pathways interacted since previous studies have also identified calcineurin as a proteolytic target of activated calpain (Shioda et al., 2006; Wu et al., 2004). While it has been separately shown that a low-amplitude [Ca\(^{2+}\); rise involves calcineurin activity (Lautermilch and Spitzer, 2000), and a high [Ca\(^{2+}\); rise involves calpain activity (Robles et al., 2003), no study to date has examined a potential
interaction between these pathways following signaling from a guidance cue. We find that upon Sema5B stimulation, calpain can cleave calcineurin, producing 45 kDa and 48 kDa constitutively active fragments. Whether this cross-talk contributes to collapse in normal cultures is unclear, however it does provide a redundant mechanism of calcineurin activation to possibly ensure the collapsing event is successful. We thus show for the first time that both calpain and calcineurin can be activated by the same stimulus, and propose that cross-talk between these effector molecules occurs (Fig. 4.8). This model therefore suggests upon activation by the same stimulus, events affecting adhesion and cytoskeletal dynamics may begin to be teased out, with calpain being more promiscuous in that it may potentially modulate adhesion and, through cleavage of calcineurin, cytoskeletal behaviour as well.

Based on our results, we propose a novel molecular mechanism for growth cone collapse stimulated by an inhibitory cue. Specifically, calpain- and calcineurin-mediated pathways may be activated in combination, with cross-talk between one another potentially occurring. Downstream targets of each pathway may modulate different components of the growth cone, the sum total of these events leading to growth cone collapse.
Figure 4.8. Model of the signal transduction cascades by which increases in $[\text{Ca}^{2+}]_i$ via Sema5B-mediated growth cone collapse act. Both calcineurin and calpain have been identified as downstream targets of Sema5B, with a calcineurin-mediated signal transduction pathway possibly signaling to the actin cytoskeleton, while a calpain-mediated signaling cascade may impinge its effects on focal contacts. Furthermore, active calpain can also induce cleavage of calcineurin producing constitutively active fragments.
Sema5B

Ca$^{2+}$

Ca$^{2+}$

Calcineurin

Calpain

Proteolysis

Growth cone collapse
4.5 References


5. GENERAL DISCUSSION

The studies presented in this thesis contribute to the growing knowledge base of the intracellular mechanisms responsible for growth cone collapse. Specifically, I have utilized a candidate collapsing compound, MotC, to elucidate potential signaling pathways involved in growth cone collapse, and then examined these effector targets under more physiological conditions using the *in vivo* inhibitory cue, Sema5B. Based on these findings, I propose each inhibitory cue requires the involvement of multiple pathways downstream of Ca\(^{2+}\) to act in a combined effort to mediate the growth cone collapse response.

5.1 Utilization of MotC as a research tool to identify pathways implicated in growth cone collapse

Neuronal outgrowth is a fundamental process for normal development of the nervous system. Despite recent advances, the molecular mechanisms regulating outgrowth, and specifically signal transduction mechanisms underlying growth cone collapse stimulated by inhibitory cues, are still poorly understood. In chapter 2, insight into this was provided by initially characterizing the effects of the candidate inhibitory compound MotC, which had previously been identified for its anti-motility effects on transformed cells. MotC was shown to be a robust inhibitor of neurite outgrowth that induced growth cone collapse and neurite retraction. This effect is reversible as after removal, neuronal growth cones re-extended lamellipodial and filopodial processes and
re-established motility. Based on the collapse phenotype, combined with the finding by McHardy et al. (2004) that MotC activates Rho signaling, I hypothesized the GTPase Rho was activated following MotC addition to DRGs. Indeed, neurons exposed to MotC exhibited a significant upregulation of active Rho-GTP. Additionally, effector-blocking experiments using Rho and Rho-associated kinase inhibitors indicated that the Rho pathway plays a critical role in this collapse response. This is therefore in agreement with other inhibitory cues also shown to upregulate this pathway (Niederost et al., 2002; Wang et al., 2002; Whitford and Ghosh, 2001; Zhang et al., 2003). For instance, Zhang et al. (2003) showed that LPA-induced neurite retraction was a result of Rho activation, which in turn was found to drive myosin-based contraction of actin filaments. Moreover, Sema4A was found to induce growth cone collapse in a Rho-ROCK-dependent manner (Yukawa et al., 2005), while MAG has also been shown to stimulate Rho activity through PKC (Domeniconi et al., 2005; Sivasankaran et al., 2004). In Chapter 2, I found inhibition of this pathway led to partial attenuation of MotC-mediated outgrowth inhibition, suggesting other pathways may be involved.

To further identify potential pathways involved in MotC-mediated inhibition of motility, chapter 3 examined whether the second messenger Ca\(^{2+}\) was involved in MotC-mediated collapse since previous experiments in Swiss 3T3 cells found high-amplitude Ca\(^{2+}\) transients were generated following MotC application (J. Church, unpublished). Similarly, in DRGs I found MotC induced a high-amplitude rise in [Ca\(^{2+}\)]. Interestingly, Robles et al. (2003) demonstrated that induction of asymmetric high intracellular Ca\(^{2+}\) transients could activate calpain on the side of the growth cone experiencing these transients, resulting in collapse on this side of the growth cone. In addition, calpain is
known to proteolytically regulate a number of signaling molecules associated with focal
contacts, including tyrosine kinases (Franco et al., 2004; Huttenlocher et al., 1997).
Robles et al. (2003) examined the involvement of calpain in this context and provided
evidence that calpain activation regulates integrin-mediated adhesion by inhibiting Src
activity, a kinase involved in the coupling of the actin cytoskeleton to the substratum
(Suter and Forscher, 2001). Other studies of calpain playing a role in adhesion have also
been demonstrated. For example, in neutrophils, which similar to neurons lack classic
focal adhesions, inhibition of calpain results in an acceleration in migration (Lokuta et al.,
2003). Another target for calpain-mediated regulation of adhesion is the cytoskeletal
protein talin-1. Calpain cleavage of talin-1 has been shown to result in a disassociation
between integrins and the actin cytoskeleton, leading to adhesion disassembly (Franco et
al., 2004).

Based on the findings by Robles et al. (2003), in combination with the high-
amplitude \([\text{Ca}^{2+}]_i\), evoked by MotC, I hypothesized that calpain activity was activated
during MotC-mediated growth cone collapse. Utilizing a fluorescence calpain reporter,
calpain activity was found to be upregulated and that inhibition of calpain results in
attenuation of MotC-mediated collapse. Though consistent with the findings from Robles
et al. (2003), these results initially appear to be in contradiction with the motile role
calpain plays in certain non-neuronal cells. Inhibition of calpain in non-neuronal cells
expressing classic adhesion complexes has been shown to reduce forward migration
(Franco et al., 2004; Huttenlocher et al., 1997; Palecek et al., 1998). These conflicting
results may be attributed to differences in the motile machinery in neuronal versus non-
neuronal cells. In the case of non-neuronal cells, forward migration requires a cyclical
balance between adhesion to the substratum at the leading edge and de-adhesion of the trailing edge (Cox and Huttenlocher, 1998; Ridley et al., 2003), while in neurons, only adhesion at the leading edge of the growth cone appears to be the critical step (Jay, 2000; Wen and Zheng, 2006). Interestingly, 16 isoforms of calpain have been identified in non-neuronal cells, the best-studied of which are calpain-1 (also referred to as -μ) and -2 (also referred to as -m). While the subcellular localization of these calpain isoforms are variable, in non-neuronal cells calpain-2 is thought to play a role in de-adhesion of the trailing edge during forward migration (Franco et al., 2005; Franco et al., 2004; Palecek et al., 1998). By specifically examining rear detachment rates of migrating cells, Palecek et al. (1998) found that inhibiting calpain prolonged rear detachment and in turn slowed forward migration. Supporting the hypothesis that calpain was involved in the uncoupling of the actin cytoskeleton from the substratum, Franco et al. (2004) showed that calpain-2 can proteolyze talin-1 at the trailing edge. In neurons, it is unclear whether select calpain isoforms localize to specific regions of the growth cone (Franco et al., 2004; Glading et al., 2002), though initial staining of calpain-1 by Robles et al. (2003) has been found to localize at the leading edge. Nevertheless, since no trailing edge exists in the motile growth cone, calpain-mediated de-adhesion from the substratum may simply promote growth cone collapse and retraction. Therefore, the seemingly contrary role of calpain function may reflect the differences in motile mechanisms employed by different cell types.

Other studies have also shown the actin binding protein vinculin to be highly enriched at adhesion complexes (Chen et al., 2005) and generation of traction forces have been directly correlated with its accumulation in fibroblasts (Hu et al., 2007), suggesting
a role for vinculin in coupling adhesion sites to the actin cytoskeleton (Ziegler et al., 2006). In agreement with this, Robles et al. (2003) showed that following inhibition of tyrosine kinase activity, and presumably outgrowth, vinculin levels were significantly reduced. Interestingly, Mchardy et al. (2004) demonstrated that following addition of MotC to either Swiss 3T3 mouse fibroblasts or MDA231 breast cancer cells, vinculin accumulation increased which reflected a rise in focal adhesion formation. Since MotC induces calpain activation in growth cones, one would predict based on the findings by Robles et al. (2003) that as a result of calpain upregulation, vinculin levels would decrease. A reason for these seemingly counterintuitive results may again be due to differences in mechanisms of motility between cell types. In the case of MotC-activated Rho in neurons, de-adhesion from the substratum is presumably required for growth cone collapse and neurite retraction (Nikolopoulos and Giancotti, 2005; Schlaepfer and Mitra, 2004; Zampieri and Chao, 2006). This de-adhesion would thus be in accordance with decreased vinculin levels. However, in non-neuronal cells, MotC-activation of Rho induces stress fiber formation and focal adhesion formation as observed by the increase in vinculin accumulation, and thus presumably contributing to the non-motile phenotype (McHardy et al., 2004; Wozniak et al., 2004). Therefore, given the differences underlying the mechanisms associated with outgrowth inhibition between neuronal and non-neuronal cells, the differences in vinculin staining observed by Mchardy et al. (2004) and Robles et al. (2003) are in line with the morphological changes associated with these cell types.

A question that arises from the findings by Mchardy et al. (2004), Robles et al. (2003), and the results presented in chapter 3 of this thesis, is whether MotC is also regulating calpain activity in non-neuronal cells. Since active calpain could target
proteolytic regulation of focal adhesions in non-neuronal cells, one would again predict a
decrease in vinculin staining if MotC in fact does upregulate calpain activity in non-
euronal cells. This result would thus be counterintuitive to my working model. If such
was indeed the case, one explanation could simply be that MotC activates a calpain
isoform specific to neurons and/or has no motile function in non-neuronal cells.
Alternatively, one could argue that if calpain-2 is primarily active at the trailing edge of
non-neuronal cells, MotC may specifically target calpain-2. In this scenario, activated
calpain-2 could mediate de-adhesion and retraction of the trailing edge, while the
functionality of calpain-1 and adhesion at the leading edge remains intact. Evidence to
support this hypothesis is found in observations by Roskelley et al. (2001) in which it was
noted that following MotC application in a wound assay, MDA-231 breast carcinoma
cells remained attached to the substratum in a rounded phenotype. This observation is
consistent with a model in which calpain-2-mediated retraction of the trailing edge
resulted in a rounded phenotype following MotC addition.

5.2 Inhibitory cues evoke a combination of multiple signaling
pathways to induce growth cone collapse

While calpain is certainly necessary for collapse, inhibition of calpain only
partially attenuates the inhibitory effects of MotC. Based on these partial effects,
combined with the incomplete attenuation of MotC-mediated collapse with inhibitors of
the Rho-ROCK pathway in chapter 2, I hypothesized both calpain and Rho-ROCK
signaling act in combination to mediate MotC-induced growth cone collapse. Indeed,
inhibition of both Rho-ROCK- and calpain-mediated pathways simultaneously had additive effects in attenuating the collapsing effects to MotC. This suggests these pathways act in combination to modulate the inhibitory effects of MotC. I also observed [Ca\textsuperscript{2+}]\textsubscript{i} was unaffected by co-application of MotC and the ROCK inhibitor, Y27632, placing the Rho-ROCK pathway downstream of Ca\textsuperscript{2+} signaling. When examined in combination with the observations that extracellular Ca\textsuperscript{2+} chelation with EGTA can abolish the growth cone collapsing effects of MotC, I proposed that induction of high-amplitude increases in [Ca\textsuperscript{2+}], also stimulates Rho-ROCK signaling in addition to calpain signaling.

While previous studies have independently examined the requirement of either Rho-ROCK- or calpain-mediated signaling mechanisms in neurons (Niederost et al., 2002; Yukawa et al., 2005; Zhang et al., 2003), no study to date has shown combined involvement. It was demonstrated in chapter 3 that both Rho-ROCK- and calpain-mediated signaling contributed additively to MotC-mediated growth cone collapse. In non-neuronal cells, evidence for the combined involvement of Rho-ROCK and calpain signaling initiated by cues influencing motility has recently been reported. Smith et al. (2006) have shown in epidermal growth factor-stimulated motile fibroblasts that both Rho-ROCK- and calpain-mediated signaling was critical for generation of contractile forces and motility. Based on the association of Rho-ROCK signaling with the actin cytoskeleton and calpain signaling with adhesion, evidence suggests a model in which growth cone collapse induced by an extracellular cue requires both the uncoupling of the cytoskeleton from the substrata and the retraction of actin filaments. Such a disruption of the link between the cytoskeleton and the substratum would negatively regulate growth
cone motility, a theory that would be consistent with the clutch hypothesis for outgrowth (Lin et al., 1996; Lin and Forscher, 1995; Mitchison and Kirschner, 1988; Sheetz et al., 1998).

Taken together, the results from chapters 2 and 3 show for the first time that both Rho-ROCK- and calpain-mediated pathways can contribute to a single growth cone collapsing event induced by an inhibitory cue. In addition, as turnover of adhesion complexes is often associated with calpain activity and retraction of the actin cytoskeleton is stereotypically associated with Rho-ROCK signaling, I propose a model of collapse that requires calpain-mediated de-adhesion of the growth cone from the substratum and Rho-ROCK-mediated contraction of actin filaments in the P-domain.

5.3 Application of pathways identified in MotC-mediated growth cone collapse using a physiological in vivo guidance cue, Sema5B

One caveat with the growth cone collapse pathways identified with MotC is that this is an organic molecule not present in vertebrates. Therefore, I sought to examine whether similar signal transduction mechanisms were stimulated by vertebrate in vivo guidance cues. The inhibitory cue Sema5B was chosen because it is an endogenous cue that stimulates chick DRG growth cone collapse and its downstream signaling pathway(s) have yet to be determined. Although Ca\(^{2+}\) has long been established to play an important role as a second messenger in growth cone motility, it has more recently been proposed that it is specifically the [Ca\(^{2+}\)] profile that regulates the signaling cascades to determine the motile behaviour of the growth cone. It has been put forth that low- and high-
amplitude rises in [Ca$^{2+}$], result in the activation of the neurite outgrowth inhibiting proteins calcineurin and calpain, respectively. However, it remains unknown if and how these mechanisms are modulated by specific guidance cues. In chapter 4, the inhibitory cue Sema5B was shown to induce growth cone collapse in chick DRGs by promoting the influx of extracellular Ca$^{2+}$. The resulting rise in [Ca$^{2+}$] was characterized by an initial low-amplitude increase followed by a marked secondary rise. Based on this multi-amplitude Ca$^{2+}$ rise, combined with previous studies suggesting low-amplitude increases in [Ca$^{2+}$] activated calcineurin signaling while high-amplitude rises activated calpain, I hypothesized both calcineurin and calpain were involved in Sema5B-mediated growth cone collapse. In support of this hypothesis, inhibition of either calcineurin or calpain attenuated Sema5B-induced growth cone collapse, an effect that was augmented by the simultaneous inhibition of both signaling cascades. Furthermore, it was shown that following the addition of Sema5B, cross-talk between these two pathways via calpain-mediated cleavage of calcineurin occurred. I thus concluded that downstream Ca$^{2+}$ targets are largely dependent on the [Ca$^{2+}$] profile generated and that this profile can be shaped by the contribution of individual guidance cues, the extracellular environment, and the intracellular environment.

In light of the findings from chapter 3 identifying calpain involvement in MotC signaling, the observation of calpain also playing a role in Sema5B-mediated growth cone collapse further highlights the essential role this protease plays during outgrowth inhibition. Based on the aforementioned evidence supporting calpain's role in regulating adhesion during growth cone motility, I find it reasonable to assume a similar role in Sema5B signaling. In addition to calpain, combined involvement of calcineurin was
identified. Active calcineurin has been shown to target GAP43 and protein phosphatase 1 (PP1), both of which have been implicated in regulating growth cone motility (Lautermilch and Spitzer, 2000; Liu and Storm, 1989; MacKintosh and MacKintosh, 1994). Active PP1, which is regulated by the inhibitor-1 (IN-1), has been found to promote actin depolymerization through dephosphorylation of actin depolymerizing factor/cofilin (Meberg et al., 1998). Moreover, active GAP43 has previously been shown to be involved in actin polymerization via uncapping of actin filaments and is found localized in growth cones (Dent and Meiri, 1998; He et al., 1997; Liu and Storm, 1989). Lautermilch and Spitzer (2000) have shown Ca\(^{2+}\)-mediated calcineurin results in dephosphorylation and inactivation of GAP43. More recently, it has been found that inhibition of either ROCK or myosin activity can attenuate Sema5B-mediated growth cone collapse (T. O’Connor, unpublished). One could thus hypothesize that Rho-stimulated myosin contraction may also contribute to this collapse response. Such a possibility would be consistent with the data published by Zhang et al. (2003) in which LPA-mediated growth cone collapse and retraction was suggested to be primarily dependent upon regulation of myosin activity. Therefore, in addition to the possibility of calpain-mediated regulation on adhesion dynamics, calcineurin- and Rho-mediated signal transduction pathways targeting the actin cytoskeleton appear to play a significant role in the collapse response stimulated by Sema5B.
5.4 Sema5B activates both calcineurin- and calpain-mediated pathways during growth cone collapse

The finding that both calcineurin and calpain act in combination to mediate Sema5B-induced growth cone collapse is of significant interest. The Ca\(^{2+}\)-set point hypothesis is well supported by experimental evidence suggesting low- and high-amplitude rises in [Ca\(^{2+}\)]\(_j\) result in an inhibitory motile response, while a mid-amplitude [Ca\(^{2+}\)]\(_j\) rise results in outgrowth (al-Mohanna et al., 1992; Gu et al., 1994; Henley and Poo, 2004; Lankford and Letourneau, 1991). Recently, Gomez and Zheng (2006) have expanded on this model by proposing that specific signaling cascades are activated in accordance with these different Ca\(^{2+}\) signals. Specifically, they proposed calcineurin-mediated pathways are activated by low-amplitude rises in [Ca\(^{2+}\)]\(_j\), CaMKII- and PKC-mediated pathways are induced by mid-amplitude increases, and calpain-mediated pathways are stimulated by high-amplitude rises. One caveat to such a generalization is that a majority of the data contributing to the identification of the downstream effectors in this model were ascertained from studies that directly modulated the levels of intracellular Ca\(^{2+}\). Therefore, whether such mechanisms are relevant to physiological in vivo guidance cues had yet to be tested.

In the case of the chemoattractant netrin-1, it may indeed fit this model. Previously, it was shown that netrin-1 induced a mid-amplitude rise in [Ca\(^{2+}\)]\(_j\), (Hong et al., 2000; Wang and Poo, 2005) and that direct local elevation of [Ca\(^{2+}\)]\(_j\) to produce a mid-amplitude increase resulted in attractive growth cone turning that was dependent upon CaMKII activation (Wen et al., 2004). Interestingly, Jin et al. (2005) provided
evidence suggesting PKC may also be a downstream target of netrin-1 signaling in cultured cortical neurons. They further showed that netrin-1 upregulated Rac activity and blocking $[\text{Ca}^{2+}]$, elevation with BAPTA-AM abolished this effect (Jin et al., 2005). This suggests a potential molecular mechanism for netrin-1-mediated attraction to stimulate a mid-amplitude rise in $[\text{Ca}^{2+}]$, which in turn activates both CaMKII- and PKC-mediated pathways, resulting in the regulation of Rho GTPases to modulate the actin cytoskeleton. Based on such a pathway, netrin-1 would be consistent with the model presented by Gomez and Zheng (2006).

However, in the case of Sema5B, I have identified an inhibitory cue capable of inducing both low- and high-amplitude increases in $[\text{Ca}^{2+}]$, and that both calcineurin- and calpain-mediated signaling cascades are activated. Moreover, I show it is the combination of these target pathways that additively contribute to the collapse response induced by Sema5B. This novel finding appears to oppose the model presented by Gomez and Zheng (2006) which suggests low- and high-amplitude $[\text{Ca}^{2+}]$-mediated pathways are independent of one another. Based on the results presented in chapter 4, I propose the $[\text{Ca}^{2+}]$ profiles generated by physiological guidance cues are much more variable and hence difficult to categorize, and as a result the downstream signaling cascades are more ambiguous. Examination of the signaling cascade initiated by other extracellular guidance cues seems to be in accord with my proposal.

In the case of the inhibitory molecule MAG, it has been shown to induce a low-amplitude rise in $[\text{Ca}^{2+}]$, via $\text{Ca}^{2+}$ release from intracellular stores to stimulate growth cone collapse (Henley et al., 2004). Based on the aforementioned model, one would predict this low-amplitude $\text{Ca}^{2+}$ response would regulate calcineurin activity. Though no
study has directly examined such a pathway, Han et al. (2007) recently showed that MAG-mediated growth cone repulsion was dependent on PP1 activity and that inhibition of PP1 resulted in conversion of repulsion to attraction. It should be noted that previous work has shown that Ca\(^{2+}\)-mediated activation of calcineurin stimulates PP1 to induce growth cone repulsion (Wen et al., 2004). Taken together, a calcineurin-mediated signal transduction cascade induced by MAG indeed seems likely. However, Sivasankaran et al. (2004) has demonstrated MAG also activates PKC, which in turn stimulates Rho activity to promote neurite retraction. Since PKC was proposed to only be involved in mid-amplitude [Ca\(^{2+}\)] responses, these results would be in contradiction with the model proposed by Gomez and Zheng (2006). In this case, a potential signal transduction cascade regulating the repulsive response of MAG would involve an initial low-amplitude [Ca\(^{2+}\)] rise, which in turn could activate both PKC-Rho- and calcineurin-PP1-mediated pathways. It should also be noted that some guidance cues have been shown to not depend on Ca\(^{2+}\). For example, Sema3A has been reported to not require Ca\(^{2+}\) to elicit its inhibitory effects (Song et al., 1998). This was further substantiated when Wen et al. (2004) demonstrated that inhibition of calcineurin or PP1 did not affect Sema3A-mediated growth cone repulsion. Clearly, the downstream targets of Ca\(^{2+}\) signaling are much more complex than previously proposed and likely depend on a multitude of conditions, including the particular guidance cue encountered, the state of the extracellular environment, and the state of the intracellular environment.
5.5 Combined activation of multiple downstream targets – a model for growth cone collapse

Based on my MotC and Sema5B studies, I conclude that the amplitude increase in [Ca\(^{2+}\)]\(_j\) specifically dictates the downstream signaling pathways mediating outgrowth inhibition and that combined activation of multiple pathways is required. I therefore propose an overall model, which is a further adaptation from the one proposed by Gomez and Zheng (2006), incorporating my findings along with past studies (Fig. 5.1). Specifically, I propose that each extracellular cue is capable of modulating multiple pathways downstream of Ca\(^{2+}\) and that these pathways likely regulate mechanisms of cytoskeletal dynamics and adhesion. For example, Sema5B activates both calcineurin- and calpain-mediated pathways during growth cone collapse, and that cross-talk between these signaling molecules occurs (Fig. 5.2). It is important to note that Figure 5.2 represents proposed signaling cascades activated by Sema5B since it has not been directly shown that the low-amplitude rise in [Ca\(^{2+}\)]\(_j\) specifically activates calcineurin while the high-amplitude rise in [Ca\(^{2+}\)]\(_j\) regulates calpain. However, evidence from Chapter 4 in which I show a time-course of calpain activation only occurring during the period corresponding to a Sema5B-induced high-amplitude [Ca\(^{2+}\)]\(_j\) rise, combined with the data in Chapter 3 in which I show calpain activation resulting from MotC-induced high-amplitude [Ca\(^{2+}\)]\(_j\) increases provides support for this model. Moreover, MotC induces collapse via activation of calpain and Rho signaling (Fig. 5.3). It would be of interest to examine what lies upstream of Rho activation during MotC signaling. Based on my model, one candidate molecule would be PKC since it has been shown to directly
Figure 5.1. Schematic summarizing signal transduction cascades implicated in different amplitude-rises in $[\text{Ca}^{2+}]_i$. Varying sizes in $\text{Ca}^{2+}$ schematic balloons represent varying amplitudes in $[\text{Ca}^{2+}]_i$ required to stimulate a particular pathway. Red schematic balloons indicate effector molecules generally involved in growth cone collapse, blue schematic balloons represent effector molecules generally associated with outgrowth, and green schematic balloons signify effector molecules generally known to promote both growth cone collapse and outgrowth.
Figure 5.2. Schematic of proposed intracellular pathways implicated in Sema5B-mediated growth cone collapse. Potential pathways regulated by Sema5B indicated by black arrows. Red schematic balloons indicate target proteins generally involved in growth cone collapse, blue schematic balloons represent targets generally associated with outgrowth, and green schematic balloons signify effectors generally known to promote both growth cone collapse and outgrowth.
Figure 5.3. Schematic outlining the potential downstream targets implicated in MotC-mediated growth cone collapse. Pathways involved in MotC signaling in neurons shown by black arrows. Red schematic balloons indicate proteins generally involved in growth cone collapse, blue represent targets typically associated with outgrowth, and green highlights effectors generally known to promote both growth cone collapse and outgrowth.
regulate Rho activity (Domeniconi et al., 2005; Sivasankaran et al., 2004) and appears to be a fairly promiscuous effector molecule involved in growth cone motility (Jin et al., 2005; Xiang et al., 2002). In the case of MAG, it has been shown to induce both PKC- and calcineurin-mediated pathways (Fig. 5.4), while netrin-1 can promote activation of PKC- and CaMKII-mediated pathways (Fig. 5.5). Furthermore, it is important to note that it has previously been proposed that the balance between phosphatase and kinase activity downstream of Ca\(^{2+}\) signals controls protrusion or collapse responses (Gomez and Zheng, 2006; Wen et al., 2004). However, based on the seemingly contradictory evidence of PKC involvement in both outgrowth promoting and inhibitory roles, it appears such a model would be too simplistic. One explanation for such a contradictory role for PKC is that specific isoforms (both typical and/or atypical) could be activated during a particular [Ca\(^{2+}\)] profile generated by a cue influencing motility. For example, since PKC has been shown to directly activate Rho to induce neurite retraction (Conrad et al., 2007; Jin et al., 2005; Luo, 2002), it is plausible that the high-amplitude [Ca\(^{2+}\)] rise induced by MotC activates a specific PKC isoform sensitive to high Ca\(^{2+}\) signals, and in turn stimulates Rho activation. Future examination of such possibilities will be required to clarify this.

I also demonstrate the importance of calpain regulation in MotC- and Sema5B-induced growth cone collapse. While evidence is provided to suggest calpain may target adhesion complexes, one must acknowledge the large array of downstream targets of calpain and therefore cannot rule out other possibilities. However, the role this protease plays in regulating adhesion, and thus motility, is well characterized (Franco et al., 2004; Gomez and Zheng, 2006; Huttenlocher et al., 1997; Robles et al., 2003). Finally, the pathways proposed to be modulated by the guidance cues in these figures have been
Figure 5.4. Schematic diagram showing the signaling cascades involved in MAG-mediated growth cone collapse. Pathways activated by MAG signaling in neurons shown by black arrows. Red schematic balloons represent proteins generally associated with growth cone collapse, blue shows targets typically associated with outgrowth, and green indicates effectors that can promote both growth cone collapse and outgrowth.
Figure 5.5. Schematic outlining the pathways regulated in netrin-1-mediated neurite outgrowth. Pathways involved in netrin-1 signaling in neurons shown by black arrows. Red schematic balloons indicate proteins generally involved in growth cone collapse, blue represent targets typically associated with outgrowth, and green highlights effectors generally known to promote both growth cone collapse and outgrowth.
represented based on the current data in the field. Though less stringent than that previously proposed (Gomez and Zheng, 2006; Kater and Mills, 1991), one still cannot exclude the possibility that cross-talk with other pathways exists. In support of this, cross-talk does in fact occur during Sema5B-mediated growth cone collapse via calpain cleavage of calcineurin, presumably yielding active fragments (Uchino et al., 2003; Wu et al., 2004). Despite this interaction, one still cannot exclude, for example, the possibility that an inhibitory signal to repress CaMKII activity occurs. I believe such complex cross-talk may be stimulated in other guidance cues as well and will require future examination to tease out these intricacies. In addition, the intimate relationship between cAMP and Ca$^{2+}$ under physiological conditions further confounds the regulation of these pathways. Since the activity of both second messengers positively feedback to one another, future studies will be required to identify how these positive feedback loops impact the aforementioned pathways. One must also acknowledge that certainly exceptions to such a general model exist and that such exceptions may be due to differences in cell types, experimental paradigms, or both.

5.6 Potential mechanisms utilized by cues influencing motility to regulate adhesion in a calpain-independent manner

As previously discussed, a cyclical process of adhesion/de-adhesion and extension/retraction is required for growth cone motility as per the clutch model (Mitchison and Kirschner, 1988). With the effects of calpain proteolysis presumably targeting adhesion complexes, one question that arises is why do certain cues such as
netrin-1 and MAG appear to not require calpain-mediated proteolytic activation? An obvious explanation is that netrin-1 and MAG regulates adhesion complexes through calpain-independent mechanisms. Certainly, previous studies have identified each guidance cue in playing a role in modulating adhesion to the substratum.

In the case of netrin-1, Li et al. (2004) have shown that the netrin receptor Deleted in Colorectal Cancer (DCC) can directly interact with Src and FAK. In the presence of Src inhibitors, netrin-stimulated axon outgrowth was attenuated (Li et al., 2004), suggesting Src-mediated adhesion is important in netrin signaling. In regards to MAG, it has been demonstrated that the MAG co-receptor LINGO-1 regulates the Src family member Fyn (Mi et al., 2004; Mi et al., 2005). Dominant-negative treatment of LINGO-1 resulted in a two-fold increase in Fyn protein and phosphorylation levels, again suggesting a role for the Src family in regulating adhesion during MAG signaling of growth cone motility (Mi et al., 2005). These studies provide evidence that both netrin-1 and MAG may regulate adhesion in a calpain-independent fashion via a more direct Src-mediated manner. Future studies will be required to test this and also to definitively show that each of these cues can simultaneously activate both actin cytoskeleton- and adhesion-mediated pathways during a growth cone advancing/collapsing event to further corroborate with my proposed model. While the model put forth by Gomez and Zheng (2006) was the result of elegant studies that directly modulated intracellular Ca\textsuperscript{2+} levels and allowed for the identification of specific pathways associated with distinct Ca\textsuperscript{2+} responses, the lack of extracellular cues to corroborate these findings with physiological growth cone behaviour may have resulted in a simplified model. My proposed model suggests that cues require combined activation of multiple downstream pathways, likely impacting that regulation
of both cytoskeleton dynamics and adhesion, to generate a motile response and that cross-talk between these signaling cascades likely occurs. Taken together, I find my proposed model to be well supported by experimental evidence, but acknowledge future studies will be required to define the intracellular complexities modulated by each cue.

5.7 Involvement of microtubules in MotC- and Sema5B-mediated growth cone collapse

While the focus of this thesis and my proposed model has been on signaling cascades targeting the actin cytoskeleton and adhesion, one cannot exclude the possibility that these inhibitory cues, including MotC and Sema5B, also impinge on microtubule organization. Previous studies examining LPA have shown that this inhibitory cue binds to its EDG family of receptors and activates both Rho-ROCK and GSK-3β-Tau signaling, leading to neurite retraction by modulating the actin and microtubule cytoskeleton, respectively (Hirose et al., 1998; Sayas et al., 2002; Sayas et al., 1999; Zhou et al., 2004). Furthermore, it has been shown that activation of GSK-3β in LPA signaling may also be mediated via RhoA (Sayas et al., 2002). Based on this data, I examined whether MotC or Sema5B regulated microtubule organization in a GSK-3β-dependent manner. Using a growth cone collapse assay, I found pharmacological inhibition of GSK-3β partially attenuated the growth cone collapsing effect of Sema5B, but not MotC (Fig. 5.6). This shows MotC is not dependent on GSK-3β signaling to mediate growth cone collapse, and suggests that microtubule re-organization does not play a primary role in mediating the inhibitory response to MotC. In the case of Sema5B, my data indicates this inhibitory
Figure 5.6. The collapsing effect of Sema5B, but not MotC, is significantly attenuated in the presence of the GSK-3β inhibitor, SB216763. Quantification of growth cone collapse expressed as percent collapsed. In experimental cultures, chick DRGs were pre-incubated with SB216763 for 20 minutes, followed by Sema5B or MotC application for 1 hour. n = 50 growth cones scored per experimental condition with experiment done in triplicate. Error bars = standard error of the mean.
guidance cue mediates GSK-3β signaling which in turn may modulate microtubule organization during the collapse response. However, future studies will be required to examine whether the involvement of GSK-3β in our system specifically modulates microtubule dynamics. Furthermore, experiments to define the relationship between this pathway and those already identified for Sema5B-mediated growth cone collapse would be required. In particular, does GSK-3β in fact signal through Tau or APC to mediate microtubule re-organization following Sema5B application? Since the Rho-ROCK pathway has been implicated in Sema5B signaling, does cross-talk to GSK-3β occur? Finally, does a hierarchy exist between adhesion and the actin and microtubule cytoskeleton that is required to initiate and carry out growth cone collapse?

5.8 Conclusion

The mechanisms regulating growth cone motility are complex and multi-factorial. They depend in a large part on the cue stimulating the motile response, as well as the intracellular and extracellular states of the neuron. This is further confounded by the spatial and temporal aspects regulating the dynamic state of the neuron. In this thesis, I present evidence that inhibitory cues, and in particular the Ca^{2+} profile generated, play a major role in determining the specific downstream targets that mediate a particular motile response. It is the hope that the work presented in this thesis contributes to furthering the knowledge base of the underlying mechanisms associated with growth cone motility.
5.9 References


