Cellular Mechanisms Underlying Retinoic Acid-Induced Growth Cone Guidance During Neuronal Regeneration

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Abstract

During the period of neuronal development, neurons must make correct synaptic connections with their appropriate targets. The intricate connections of the nervous system are established in part by growth cones, located at the tips of extending neurites. These unique structures are essential for axon pathfinding and target cell selection by sensing and integrating numerous guidance cues from their environment. Retinoic acid, the active metabolite of vitamin A, is an important regulator of neurite outgrowth during vertebrate development, but there is substantial evidence that it also plays a role in axon guidance. Previous studies have provided preliminary evidence of a potential role of retinoid receptors in mediating the chemotropic effects of retinoic acid. In this study, I demonstrated that a synthetic retinoic acid receptor agonist was able to mimic the effects of retinoic acid in inducing growth cone turning. I also examined the intracellular pathways activated by retinoic acid that induce changes in growth cone behaviour. Previously it has been shown that retinoic acid-induced growth cone turning of invertebrate motorneurons requires local protein synthesis and calcium influx, similar to other known guidance cues in the central nervous system. However, the signalling pathways that link calcium influx to the regulation of cytoskeletal dynamics involved in growth cone turning are not currently known. Here, I examined potential effectors downstream of retinoic acid and have provided evidence that the intracellular pathways likely involve the Rho GTPases, Rac and Cdc42. I demonstrated that the inhibition of Rac or Cdc42 prevented growth cone turning towards retinoic acid. However, it was shown that the involvement of Rac differed depending on whether the growth cones maintained communication with the cell body or not. Moreover, the inhibition of Cdc42 not only blocked growth cone turning towards retinoic acid, but also

induced a switch in growth cone responsiveness from attraction to repulsion. Overall, these studies provide new knowledge of the mechanisms underlying growth cone pathfinding by retinoids during nervous system development and regeneration.

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List of Abbreviations

ABS	Antibiotic saline
ADH	Alcohol dehydrogenase
Arp2/3	Actin-related protein 2/3
atRA	All-*trans* retinoic acid
BDNF	Brain-derived neurotrophic factor
CaMKII	Calcium-calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
Cdc42	Cell division control protein 42
9-*cis* RA	9-*cis* retinoic acid
CM	Conditioned medium
CNS	Central nervous system
CRABP	Cellular retinoic acid-binding protein
CYP26	Cytochrome P450 enzyme
DM	Defined medium
DMSO	Dimethyl sulfoxide
EtOH	Ethanol
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitors
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine triphosphate
GTPase	Guanine triphosphatase

*Lym*RAR	*Lymnaea* retinoic acid receptor
*Lym*RXR	*Lymnaea* retinoid X receptor
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline (TWEEN)
PeA	Pedal A
PKC	Protein kinase C
Rac	Ras-related C3
RALDH	Retinal dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBP	Retinol-binding protein
Rho	Ras homolog
RNA	Ribonucleic acid
ROCK	Rho kinase
RXR	Retinoid X receptor
STRA6	Stimulated by retinoic acid gene 6
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family verprolin homology

Chapter 1:

Introduction and Literature Review

1.1 General introduction

Our nervous system consists of billions of neurons interconnected to form intricate neural networks. The establishment of correct connections during the period of development is fundamental for the proper functions of the nervous system. Formation of neural circuits involve multiple developmental events including cell proliferation, differentiation, neural migration, growth cone formation, growth cone guidance and synaptogenesis. While each stage is essential for the formation of functional neural networks, in this thesis I will specifically be focusing on growth cone guidance. Growth cones are structures located at the tips of extending neurites and are essential for axon pathfinding and target cell selection, by sensing and responding to numerous guidance cues in their surrounding environment. Guidance signals are transduced through intracellular signalling pathways and converge onto the cytoskeleton to modify the behaviour of the growth cone, causing it to turn towards or away from the signal.

Retinoic acid is a critical signalling molecule that mediates neurite outgrowth and importantly, can direct neurite outgrowth by inducing positive growth cone turning (Dmetrichuk et al., 2006). Although much is known about the ability of retinoic acid to mediate neurite outgrowth during nervous system development, the intracellular pathways activated by retinoic acid that regulate growth cone behaviour are not well understood. The main goal of my research is thus to determine the cellular and molecular mechanisms by which retinoic acid mediates neurite guidance and growth cone turning. Insights derived from these studies will enhance our basic understanding of neuronal pathfinding during nervous system development.

1.2 Retinoic acid

Retinoic acid is the biologically active product of Vitamin A (retinol). Vitamin A cannot be synthesized directly; instead it must be obtained from the dietary intake of plants in the form of β-carotene, or from animal meat in the form of retinyl esters (Simões-Costa et al., 2008). These compounds are stored in the body, particularity in the liver, as retinyl esters (known as retinoids) until needed (Maden, 2007). Retinoids are then transported through the bloodstream by retinol-binding protein 4 (RBP4). Stimulated by retinoic acid 6 (STRA6), a membrane receptor, interacts with retinol and facilitates its uptake into target cells. Once retinol enters the cytoplasm of the cell it binds to cellular retinol-binding protein 1 (RBP1), where it is converted to retinal by alcohol dehydrogenase and then to retinoic acid by retinal dehydrogenases (RALDHs) (Maden and Hind, 2003). Following retinoic acid synthesis, cellular retinoic acid-binding proteins (CRABPs) bind retinoic acid, which transports the molecule into the nucleus (autocrine signalling) or out of the cell (paracrine signalling). In autocrine signalling, retinoic acid can enter the nucleus (assisted by CRABP2), where it binds to two classes of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), to regulate gene transcription. Retinoic acid levels are regulated by the cytochrome P450 enzyme Cyp26, which further metabolizes retinoic acid into polar metabolites (Maden and Hind, 2003; Fig. 1.1).

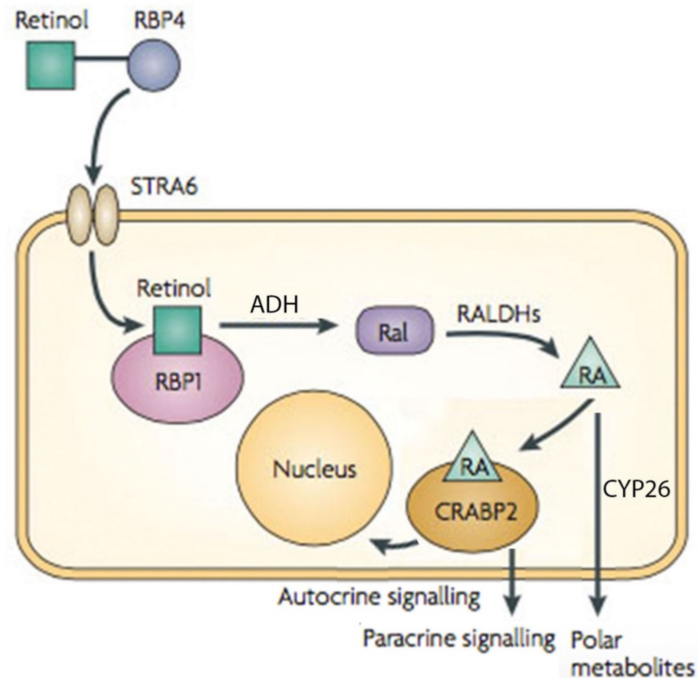


Figure 1.1: Retinoic acid synthesis. Retinol bound to retinol-binding protein 4 (RBP4) is taken into the cell by STRA6 (stimulated by retinoic acid 6). Retinol binds retinol-binding protein 1, (RBP1) and is converted to retinal (RAL) by alcohol dehydrogenase (ADH) and then to retinoic acid (RA) by retinal dehydrogenases (RALDHs). Retinoic acid binds to cellular retinoic acid-binding protein 2 (CRABP2) and can enter the nucleus. The cytochrome P450 enzyme (Cyp26) further metabolizes retinoic acid into polar metabolites. (Modified from Maden, 2007).

Modes of action of retinoic acid

Retinoic acid exerts its effects by binding to the nuclear receptors, RAR and RXR, which induce signalling and regulate gene transcription (Allenby et al., 1994). Retinoid receptors are activated by the binding of different retinoic acid isomers, 9-*cis* retinoic acid (9-*cis* RA) and all-*trans* retinoic acid (atRA). In vertebrates, both the RAR and RXR exist as three sub-types (α, β, and γ), but differ in that the RAR can bind to both atRA and 9-*cis* RA, while the RXR has been shown to preferentially bind 9-*cis* RA (Heyman et al., 1992; Egea et al., 2000). Following ligand binding, the receptors heterodimerize (or homodimerize; RXRs only) and activate retinoic acid response elements (RARE) to induce changes in gene transcription (Maden, 2007; Fig. 1.2).

RAREs are promotor DNA sequences, which most frequently consist of direct repeats (DRs), separated by 1, 2 or 5 nucleotides (DR1, DR2 and DR5, respectively). RAR/RXR heterodimers can bind to DR1, DR2 and DR5, whereas the RXR homodimers preferentially bind DR1 (Gilardi and Desvergne, 2014). Thus, RXR homo/heterodimers can compete for the same response elements which can impact the expression of target genes depending on the protein abundance of RXRs (Gilardi and Desvergne, 2014). However, the RAR/RXR heterodimer binds more efficiently to RAREs than RXR homodimers (Leid et al., 1992). Examples of RARE-associated genes include RARβ, Cyp26, CRBP1 and CRABP2 (Bastien and Rochette-Egly, 2004).

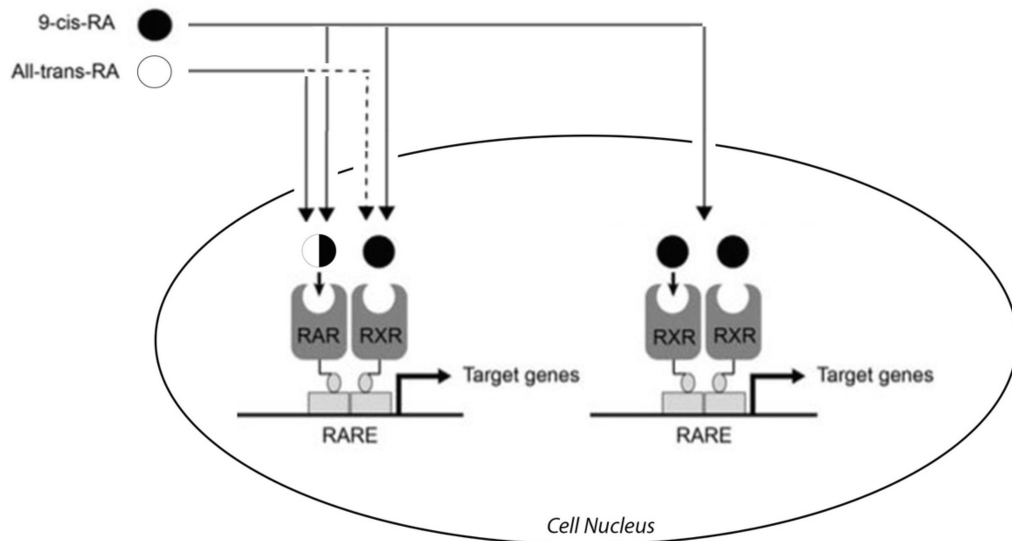


Figure 1.2: Retinoic acid signalling. In the nucleus, retinoid receptors are activated by the binding of different retinoic acid isomers. Vertebrate retinoic acid receptors (RARs) can be activated by the binding of both atRA and 9-*cis* RA, whereas the retinoid X receptors (RXRs) are preferentially activated by the binding of 9-*cis* RA, although can bind atRA at a lower affinity (indicated by dashed arrow). The receptors will then either homodimerize (RXRs) or heterodimerize and bind to retinoic acid-response element (RARE) to activate gene transcription (Modified from Simões-Costa et al., 2008).

Evidence has been provided that the biologically active isomers of retinoic acid, atRA and 9-*cis* RA, are capable of acting in both similar and differing manners. For instance, both isomers have been shown to enhance the cholinergic properties of a murine septal cell line by increasing the expression of choline acetyltransferase mRNA (Pedersen et al., 1995). Moreover, the all-*trans* and 9-*cis* retinoic acid isomers have been shown to similarly inhibit the proliferation of human neuroblastoma cells (Plum and Clagett-Dame, 1995), and induce similar chemotropic effects on *Lymnaea* neurons (Dmetrichuk et al., 2008).

At the same time, the capacity of the two isomers to act in a differing manner has also been observed. For example, 9-*cis* RA is more potent than atRA in inducing differentiation in human neuroblastoma cells (Han et al., 1995; Lovat et al., 1997). Furthermore, atRA and 9-*cis* RA have differing effects on the electrical properties and intracellular calcium levels of *Lymnaea* neurons (Vesprini and Spencer, 2014; Vesprini et al., 2015; de Hoog, 2018). That is, there is an isomer-dependent effect, where atRA is capable of reducing intracellular calcium levels and changing the firing properties of a cell, while 9-*cis* RA is not. AtRA has been identified in the nervous system of many vertebrates (Madden et al., 1998; Werner and Deluca, 2002), and is the most widely studied isomer. However, current literature lacks a clear understanding of the role of 9-*cis* RA in the nervous system.

Importance of retinoic acid during development

It is well known that retinoic acid plays an essential role in development in vertebrates. Retinoic acid initiates cellular differentiation as well as mediates patterning of

the nervous system (Zile, 1998; Maden and Hind, 2003; Maden, 2007). Retinoic acid, as well as retinoic acid synthesizing enzymes, have been detected in several species at various stages of development (Ang and Duester, 1997; Madden et al., 1998; Monaghan and Maden, 2012). Furthermore, it has been shown that either an increase or a decrease in retinoid concentrations can produce developmental abnormalities in vertebrates (Shenefelt, 1972; Vandersea et al., 1998; Gale et al., 1999). Excess retinoids result in a wide array of developmental defects including in limbs (Kochhar, 1973; Cunningham and Duester, 2016) and in heart (Chazaud et al., 1999; Nakajima, 2019), partly resulting from perturbed neural crest development (Abu-Abed et al., 2001). The effects of excess retinoids indicate the importance of controlling its distribution. Conversely, nutritional deficiencies in vitamin A have been shown to result in embryonic malformations that include abnormal heart, neural crest, eye, and nervous system development (Dickman et al., 1997). Additionally, retinoid receptor knockdown leads to similar abnormalities, providing further evidence for the importance of retinoid signalling during development (Zile, 2001).

Importance of retinoic acid during regeneration

In higher vertebrates, including humans, the regenerative capacity of the central nervous system (CNS) is extremely limited. Damage to the adult CNS often results in permanent neurological deficits due to the inability of mature axons to regenerate following injury (Yiu and He, 2006). Factors that contribute to the inability of CNS neurons to regenerate include reduced trophic support and the presence of multiple inhibitory molecules (Giger et al., 2010). In contrast to the adult mammalian CNS, some lower vertebrates, such as newts and salamanders, and invertebrates such as the mollusc *Lymnaea*

stagnalis, retain the ability to regenerate CNS neurons into adulthood (Chernoff et al., 2003; Dmetrichuk et al., 2006). Therefore, understanding the mechanisms by which these species can regenerate, may enhance our understanding of the cellular pathways involved in mammalian CNS regeneration.

Retinoic acid has been shown to play a vital role in both nervous system development and regeneration. Due to the preservation of retinoic acid in development and regeneration, it is thought that neuronal regeneration may involve the reactivation of developmental signalling pathways (Maden and Hind, 2003). Furthermore, the downregulation of retinoid signalling may contribute to the inability of mammalian CNS neurons to regenerate following injury. For example, there is evidence that retinoic acid induces regeneration of neurites in mouse embryonic neurons, but not in adult spinal cord cultures (Corcoran et al., 2002). It was thus postulated that the inability of retinoic acid to induce neurite outgrowth in the adult spinal cord is due to diminished expression of the RAR. This is indeed the case, as an overwhelming body of evidence has indicated a role for the RAR during regeneration (Corcoran et al., 2000; 2002; Puttagunta and Giovanni, 2012; Nguyen et al., 2017). For example, a specific RAR subtype, RARβ, mediates neurite outgrowth in adult newt spinal cord explants (Dmetrichuk et al., 2005). Additionally, inhibition of RARβ signalling with a selective RARβ antagonist prevents regeneration in axolotl tails (Nguyen et al., 2017).

Previous studies also support a role for retinoic acid in regeneration of limbs following amputation. In axolotls, newts, and several species of salamanders, retinoic acid induced regeneration in a concentration-dependent manner (Madden and Hind, 2003).

Moreover, retinoic acid synthesis and signalling have shown to be upregulated following injury in several species (Zhelyznik et al., 2003; Mey et al., 2005; Duprey-Diaz et al., 2016). For example, following peripheral nerve crush in adult rats, Zhelyznik et al. (2003) showed an increase in cellular retinoic acid-binding protein (CRABP2), and cellular retinol binding protein (CRBP2). Moreover, after optic nerve injury in the frog, components of the retinoid signalling pathway were upregulated, namely, retinol dehydrogenases (RALDH) and CRABPs (Duprey-Diaz et al., 2016). Additionally, in species that retain the ability to regenerate CNS tissue in adulthood, retinoic acid can promote neurite outgrowth (Dmetrichuk et al., 2005) and guide regenerating neurites (Dmetrichuk et al., 2006).

Retinoid receptor signalling is conserved between vertebrates and invertebrates

In vertebrates, both the RAR and the RXR exist as three sub-types (α, β, and γ), but they differ in that the RAR can bind both atRA and 9-*cis* RA, while the RXR has shown to preferentially bind the 9-*cis* RA isomer (Heyman et al., 1992; Egea et al., 2000). RXRs are the predominant invertebrate retinoid receptors, however, unlike the vertebrate RXRs, the RXR from the locust, *Locusta migrantoria,* has been shown to bind both atRA and 9-*cis* RA at similar affinities (Nowickj et al., 2008). This suggests that the binding affinity of retinoid receptors may differ between vertebrates and invertebrates. In the mollusc, *Lymnaea stagnalis,* the RXR (*Lym*RXR) has been cloned from the CNS and exhibits ~80% amino acid homology with the vertebrate RXRα (Carter et al., 2010). Despite the known presence of *Lym*RXR, its ability to bind retinoids has not yet been determined. However, the *Lym*RXR has an overall amino acid identity of 97% with the RXR from a closely related

mollusc, *Biomphalaria glabrata,* which has been shown to bind 9-*cis* RA (Bouton et al., 2005).

It was originally proposed that only vertebrates expressed both the RXR and the RAR, while invertebrate non-chordates expressed only the RXR. However, recent evidence suggests that the RAR is not merely a vertebrate innovation and that its role in development may be conserved between vertebrates and at least some invertebrates. Our laboratory first cloned a non-chordate protostome RAR from *Lymnaea* (*Lym*RAR; Genbank Accession # GU932671)*,* which has an overall amino acid identity of 55% with the vertebrate RARβ from *Homo sapiens*, though has similar identity with RARα and RARγ from other vertebrates, such as *Xenopus laevis*. Since then, other protostome RARs have been subsequently identified and cloned. Urushitani et al. (2013) isolated and cloned RAR-like cDNA from the rock shell, *Thais clavigera*, while Gutierrez-Mazariegos et al. (2014) characterized the RAR in the mollusc, *Nucella lapillus*. Through 3D modelling, Gutierrez-Mazariegos et al. (2014) have shown that the *N. lapillus* RAR is structurally similar to vertebrate RAR, though did not appear to bind atRA. More recently however, an RAR from the annelid *Platynereis dumerilii* has been shown to bind atRA and activate transcription (Handberg-Thorsager et al., 2018). Despite this more recent evidence for the existence of non-chordate, protostome RARs, far less is known of their physiological roles. Though the annelid RAR has been proposed to control neurogenesis and axon outgrowth (Handberg-Thorsager et al., 2018), little else is known about the physiological roles of invertebrate, protostome RARs, or their potential role in retinoid signalling during embryonic or nervous system development.

1.3 Growth cones are the essential pathfinding structures of developing and regenerating neurons

During the period of neuronal development and regeneration, neurons must make correct synaptic connections with their appropriate targets. The intricate connections of the nervous system are established, in part, by growth cones, located at the tips of extending neurites. These unique structures are essential for axon pathfinding and target cell selection by sensing and responding to numerous guidance cues in their environment. Guidance signals are transduced through intracellular signalling pathways, which act on the cytoskeleton to modify the behavior of the growth cone.

The neuronal growth cone

Growth cones are highly dynamic structures located at the leading edge of extending neurites. The neuronal growth cone was first characterized by Ramón y Cajal in 1890 (de Castro et al., 2007). Since then, extensive research has been conducted in which we are now aware that the structure of the growth cone, and its associated functions, allows for proper axon pathfinding. The growth cone is comprised of three distinct morphological domains: the central domain, transitional domain, and the peripheral domain (Fig. 1.3). The central domain consists of stable microtubules organized into bundles, as well as vesicles and organelles that support neurite extension (Vitrol and Zheng, 2012). The transitional domain is located at the interface between the central and peripheral domains where actomyosin contractile structures lie perpendicular to actin filaments (Lowery and Van Vactor, 2009). The peripheral domain is divided further into lamellipodia (veil-like projections) and filopodia (finger-like projections) that undergo protrusion and retraction

to sense their environment and generate the force required for advancing the growth cone (Forscher and Smith, 1988). In contrast to the central domain, the peripheral domain is supported by actin filaments that are organized into a mesh-like formation in the lamellipodia and parallel bundles in the filopodia (Bamburg, 2003). Actin filaments undergo polymerization and retrograde flow, which allow for protrusion and retraction of the plasma membrane respectively (Lowery and Van Vactor, 2009; Suter and Forscher, 1998). The dynamics of actin filaments are regulated by actin binding proteins which have several functions including binding/sequestering actin monomers and filament stabilization (Dent and Gertler, 2003).

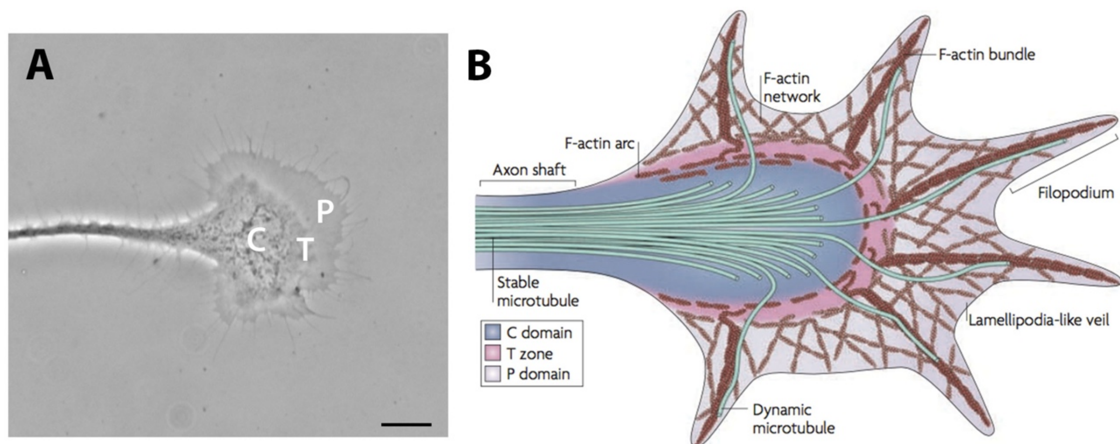


Figure 1.3: The neuronal growth cone. (**A**) Phase contrast image of a *Lymnaea stagnalis* growth cone from a Pedal A (PeA) cultured neuron. The growth cone is divided into 3 domains: the central domain (C), the peripheral domain (P), and transitional domain (T). Scale bar: 15 µM. (**B**) A schematic depicting the morphology and structure of a growth cone. The growth cone is comprised of a P-domain containing actin-based lamellipodia and filopodia, a T-domain at the interface between the P and C domains, and a C-domain enriched with bundles of microtubules. (Taken from Lowery and Van Vactor, 2009).

Growth cone guidance

There are numerous spatially and temporally distributed guidance cues that direct the migration of growing axons towards their correct targets. Membrane receptors expressed on the growth cone can be activated by guidance cues in an asymmetric manner, leading to a change in the direction of growth cone advance, which allows for highly regulated directional pathfinding. Guidance molecules that regulate growth cone pathfinding can be divided into two main categories according to their function: diffusible chemotropic cues and substrate-bound cues (Lowery and Van Vactor, 2009). Diffusible chemotropic cues are secreted factors that are released by other cells (even perhaps the same cell) and thus may guide growth cones over long distances. In contrast, substrate-bound cues serve as a physical substrate for cell attachment and axon extension. These can be located directly on the surface of neighbouring cells (e.g. cell adhesion molecules such as integrins, cadherins, axonin-1) or be part of the extracellular matrix (e.g. laminins, fibronectin) (Kiryushko et al., 2004).

Netrins, ephrins, semaphorins and slits are the most well-studied families of extracellular guidance molecules, although several other molecules have been shown to act as guidance cues, including neurotransmitters (Spencer et al., 2000), neurotrophins (Song et al., 1997), classical morphogens such as the Hedgehog and Wnt families (Charron and Tessier-Lavigne, 2005) and retinoic acid (Dmetrichuk et al., 2005). Additionally, there are other factors that influence axon guidance, such as electric fields (McCaig et al., 2005). Growth cones can turn towards the source of a guidance cue, referred to as attraction, or away from a cue, referred to as repulsion. However, whether a certain cue is attractive or repulsive can be modulated by various factors, including intracellular messengers and the

regulation of receptors. For example, studies of cultured developing *Xenopus* neurons have demonstrated that inhibition of cyclic adenosine monophosphate (cAMP) causes an attractive growth cone response to netrin to be switched to repulsion (Ming et al., 1997; Nishiyama et al., 2003). Additionally, commissural axons initially drawn to the midline, are unresponsive to the repellant slit (Zou et al., 2000). Upon crossing, commissural axons change their responsiveness to slit and are now repelled, due to the upregulation of the slit receptor Roundabout (Sabatier et al., 2004). Collectively, these studies demonstrate the influence of receptors and intracellular messengers in determining the response of a growth cone to a guidance cue.

The role of retinoic acid in growth cone guidance

Retinoic acid is an important regulator of neurite outgrowth during vertebrate development (Maden et al., 1998; Corcoran et al., 2000), but there is also substantial evidence that it acts as a guidance cue to induce positive growth cone turning (Dmetrichuk et al., 2005; 2006; Farrar et al., 2009; Carter et al., 2010). The chemotropic effects of retinoic acid to guide neurite outgrowth were first described in dissociated cultures of embryonic chick neural tissue. Using a Dunn chamber, Maden et al. (1998) demonstrated that neurites changed their orientation in response to a gradient of retinoic acid and extended towards higher retinoic acid concentrations. More recently, evidence for retinoic acid chemoattraction has been provided in cultured spinal cord explants from the newt (Dmetrichuk et al., 2005) and using cultured *Lymnaea* motorneurons (Dmetrichuk et al., 2006). In addition, it was shown for the first time that an exogenous source of retinoic acid can induce turning of individual growth cones and importantly, that the chemotropic effects

of retinoic acid are similar for both the all-*trans* and 9-*cis* isomers of retinoic acid (Dmetrichuk et al., 2006; 2008).

Local response of growth cones to guidance cues

Growth cones that are isolated from the cell body have been shown to be capable of migrating autonomously and maintaining the ability to respond to guidance cues. For example, *Xenopus* retinal axons that are severed from the soma display normal pathfinding behaviour, which may indicate a role for local protein synthesis in the growth cone (Harris et al., 1987). Local protein synthesis is a means to make required proteins on site by local mRNA translation (Kim and Jung, 2015). This process is important for growth cone pathfinding as growing axons respond to guidance cues very rapidly and often at large distances from their somas (Campbell and Holt, 2001). Several studies have shown that machinery necessary for local protein synthesis (mRNA and ribosomes) are present within the growth cones (Davis et al., 1992; Campbell and Holt, 2001; Lin and Holt 2007). Furthermore, Campbell and Holt (2001) demonstrated that isolated axons maintain their appropriate responsiveness *in vitro* to netrin-1 and Semaphorin3A, but these responses are blocked by protein synthesis inhibitors. Similar to traditional guidance cues in the CNS, retinoic acid-mediated growth cone turning does not require gene transcription (and also relies on local protein synthesis). Farrar et al. (2009) demonstrated that retinoic acid can induce growth cone turning of *Lymnaea* motorneurons isolated from the cell body and that this turning response is dependent on local protein synthesis. These studies suggest that local protein synthesis is an essential mechanism by which growth cones are able to respond to guidance cues, independently of the soma.

1.4 Environmental cues that growth cones encounter are transduced through second messenger signalling cascades

In order to understand how guidance molecules direct migration, it is necessary to study the signalling cascades that transduce external cues into directional changes. Numerous signalling pathways that are activated by guidance molecules must converge onto the cytoskeleton in order to modify the behaviour of the growth cone. The intracellular concentration of calcium is one well-established regulator of cytoskeletal dynamics, which in turn, influences the direction of neurite growth (Henley and Poo, 2004; Sutherland et al., 2014; Gomez and Zhang, 2006). Calcium acts a second messenger to convey extracellular information to produce a change in growth cone motility. Several known guidance cues within the CNS require calcium influx within the growth cone, such as netrin and brain derived neurotrophic factor (BDNF) (Ming et al., 1997; Song et al., 1997). However, the signalling cascades which link calcium influx to cytoskeletal movements require further investigation.

Rho GTPases as potential downstream effectors in growth cone signalling

The Rho GTPases, a family of small GTP binding proteins, have emerged as important regulators of the actin cytoskeleton and may link calcium changes to cytoskeletal rearrangements. Indeed, Rho GTPases serve as "molecular switches" that act through effector proteins to regulate cytoskeletal dynamics in many cellular processes (Etienne-Manneville and Hall, 2002). This family of proteins consists of Ras homolog (Rho), Ras-related C3 (Rac) and cell division control protein 42 (Cdc42), all of which are involved in growth cone responses. Indeed, many guidance molecules have been shown to regulate the

activity of Rho GTPases including slits, semaphorins, ephrins, and netrins (Wahl et al., 2000; Wong et al., 2001; Li et al., 2002; Whiteford and Ghosh, 2001; Jin et al., 2005). Furthermore, several studies have shown that loss of function of these GTPases results in axon pathfinding defects (Hall and Lalli, 2010). Specifically, genetic disruption of Rho GTPase activity results in guidance errors in *Xenopus* (Ruchhoeft et al., 1999), *Drosophila* (Ng et al., 2002), and C. *elegans* (Lundquist, 2003), suggesting that precise regulation of Rho GTPases is necessary for axon guidance. However, the distinct role of Rho GTPases in mediating axon guidance towards retinoic acid is not yet known.

Regulation of Rho GTPases

Rho GTPases cycle between an inactive, GDP-bound state and an active, GTP-bound state (Fig. 1.4). At least three known classes of proteins regulate the activity of Rho GTPases: GEFs, GAPs, and GDIs. Guanine nucleotide exchange factors (GEFs), catalyze the exchange of GDP (guanosine diphosphate) for GTP (guanosine triphosphate), which promotes Rho GTPase activation. Conversely, GTPase-activating proteins (GAPs) inactivate Rho GTPases by increasing their endogenous GTPase activity. GEFs and GAPs are more abundant and structurally complex than Rho GTPases (Dickson, 2001). This allows growth cones to integrate numerous signalling pathways and produce a single output, a change in growth cone motility. Additional regulation occurs by guanine nucleotide dissociation inhibitors (GDIs) which sequester Rho GTPases in an inactive GDP-bound conformation and restrict their spatial distribution. It is important to note that given the complexity of the signalling pathways involving Rho GTPases and the immense

number of GEFs, the specific effects of Rho GTPases depend on their localization and downstream effectors.

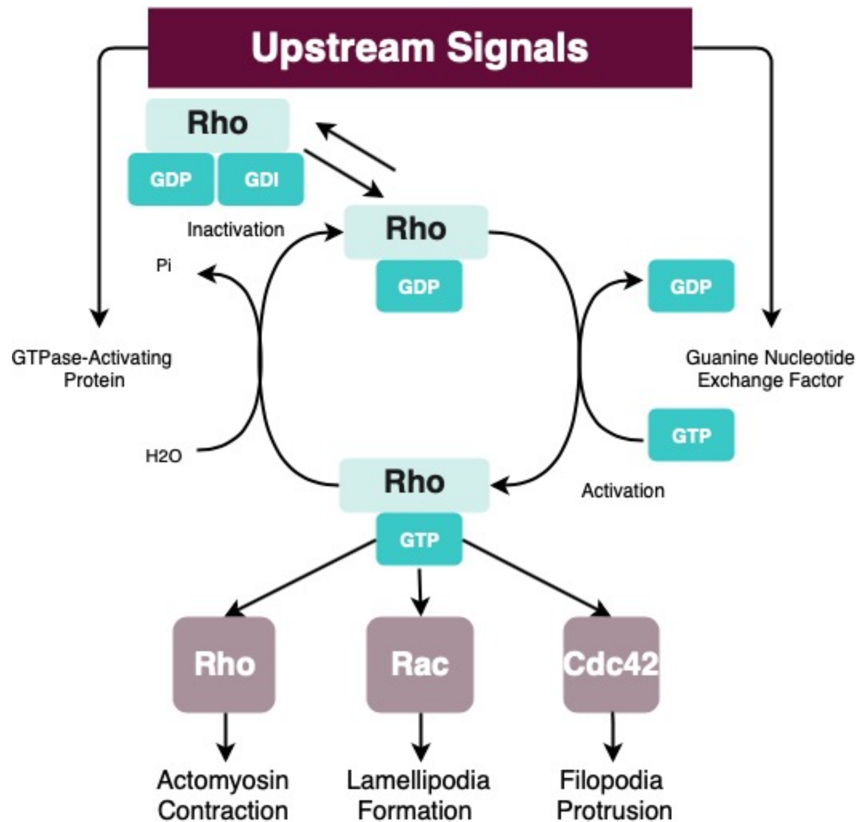


Figure 1.4: Rho GTPase function in growth cone guidance. Upstream signals are transduced to Rho GTPases through the regulation of guanine nucleotide exchange factors (GEFs) or GTPase-activating proteins (GAPs), which facilitates the activation or inactivation of Rho GTPases, respectively. Rho GTPases cycle between an active GTP-bound state and an inactive GDP-bound state. Additional regulation occurs by guanine nucleotide dissociation inhibitors (GDIs) which sequester Rho GTPases in an inactive GDP-bound conformation and restrict their spatial distribution. Activated Rho GTPases interact with downstream effectors to regulate actin cytoskeletal dynamics. The Rho GTPases, Rac and Cdc42, stimulate lamellipodia formation and filopodia protrusion, respectively. The Rho GTPase, Rho, stimulates actomyosin contraction. (Modified from Luo, 2000).

Mechanisms of actin regulation by Rho GTPases

The spatial and temporal regulation of Rho GTPases within the growth cone underlies the positive and negative turning response to guidance cues. Several studies have

suggested that Rac and Cdc42 participate in growth cone advance and attraction, whereas Rho is involved in growth cone collapse and repulsion (Dickson, 2001; Hall and Lalli, 2010). Thus, Rho GTPases are said to have opposing functions on growth cone guidance. According to the predominant model, Rac and Cdc42 are activated on the chemoattractant-facing side of the growth cone, whereas Rho is less active. Activation of Rac and Cdc42 regulate the formation of growth cone lamellipodia and filopodia, respectively (Etienne-Manneville and Hall, 2002). Effectors of Cdc42 and Rac include Wiskott-Aldrich syndrome protein (WASP) and related proteins, which promote actin formation (Govek et al., 2005). WASP proteins can be directly activated by Cdc42 and interact with the cytoskeleton through actin-related protein 2/3 (Apr2/3) complex (Machesky and Insall, 1998; Millard et al., 2004). Additional members of the WASP family, known as WASP family verprolin homology (WAVE) proteins, mediate actin cytoskeletal changes downstream of Rac. Other well-known effectors of both Rac and Cdc42 include the family of serine/threonine kinases, p21-activated kinases, whose substrates include cytoskeletal regulators such as LIM-kinase and filamin (Bokoch, 2003).

During chemorepulsion, the reverse is thought to occur where Rho is more active on the chemorepulsive-facing side of the growth cone and Rac and Cdc42 are less active. The activity of Rho is mediated by Rho-associated kinase (ROCK), which phosphorylates myosin light chain and thus results in increased actomyosin contraction (Hall and Lalli, 2010). Increased myosin activity can increase retrograde flow which results in decreased leading edge protrusion (Gomez and Letourneau, 2014). Additionally, actomyosin contraction results in growth cone collapse, or repulsion due to asymmetric growth cone collapse (Zhou and Cohan, 2001; Gomez and Letourneau, 2014).

Crosstalk among Rho GTPases

Accumulating evidence indicates that there is extensive crosstalk among Rho GTPases, though the nature of these interactions is not yet fully understood. Initially, it was proposed that Rho GTPases act in a linear pathway in which Cdc42 could activate Rac, which would in turn activate Rho (Nobes and Hall, 1995). It is not clear if a linear pathway functions in neurons as there are several studies suggesting that Rac and Cdc42 can act antagonistically to Rho, by which activation of one will result in inactivation of the other (Sander et al., 1999; Yuan et al., 2003). Ultimately, this suggests a balance among the Rho GTPases with complex interactions between the signalling pathways. Crosstalk can occur not only at the level of the Rho GTPases, but also through upstream regulators such as GEFs or GAPs, and more commonly, though downstream effectors which converge the actions of Rho GTPases on myosin activity (Yuan et al., 2003).

1.5 Use of *Lymnaea stagnalis* to study the effects of retinoids on growth cone guidance

Lymnaea stagnalis is a well-established model system to study neuronal regeneration and growth cone guidance. In contrast to higher vertebrates, *Lymnaea* neurons have the ability to grow and regenerate into adulthood (Syed et al., 1990). The CNS of *Lymnaea* contains large identifiable neurons that can be isolated from the CNS and are capable of survival and regeneration in cell culture (Syed et al., 1990). The growth cones that form at the tips of neurites from *Lymnaea* neurons in culture are large, supporting the study of growth cone behaviours. Furthermore, neurites that are severed from their soma are capable of continuing to regenerate for up to approximately 12 hours, independent of communication with the cell body (Farrar et al., 2009). Overall, *Lymnaea* provides the

opportunity to study the effects of retinoids on growth cone guidance at the cellular level in adult regenerating neurons, which would not be possible in organisms with more complex nervous systems.

Retinoid signalling in Lymnaea stagnalis

The role of retinoic acid in the developing and regenerating CNS of *Lymnaea stagnalis* has been well documented. Both all-*trans* retinoic acid and 9-*cis* retinoic acid isomers have been identified in the CNS of *Lymnaea* at similar concentrations, (though with atRA being higher than 9-*cis* RA). Furthermore, similar neurotrophic and chemotropic effects of these retinoid isomers have been demonstrated on cultured neurons from this organism (Dmetrichuk et al., 2008). Both all-*trans* and 9-*cis* retinoid isomers can induce neurite outgrowth (as they are known to do in vertebrate nervous systems) and may also mediate axon guidance, as they can induce attractive growth cone turning in regenerating motorneurons (Dmetrichuk et al., 2008; Farrar et al., 2009).

Retinoic acid exerts its main effects via signalling through receptors, RARs and RXRs. In *Lymnaea,* the RXR (*Lym*RXR) and a putative RAR (*Lym*RAR) have been cloned (Carter et al., 2010; 2015). *Lym*RXR and *Lym*RAR have been identified in the developing embryo of *Lymnaea*, and RXR and RAR agonists or antagonists have been shown to disrupt embryonic development (Carter et al., 2010; 2015; Johnson et al., 2019). Furthermore, immunostaining evidence has been provided for the presence of both the *Lym*RXR and *Lym*RAR in the adult CNS of *Lymnaea.* These receptors are localized on the neurites and growth cones of regenerating motorneurons (Carter et al., 2010; 2015). Antagonists for the RXR have been shown to block growth cone turning towards retinoic acid suggesting a

role for these receptors in axon guidance (Carter et al., 2010). Importantly, RXR agonists produce growth cone turning of growth cones isolated from the cell body (Carter et al., 2010), suggesting a non-genomic role of RXRs in retinoic acid growth cone guidance. Indeed, retinoic acid-induced growth cone turning can occur in the absence of gene transcription and requires local protein synthesis and calcium influx (Farrar et al., 2009). Additionally, a vertebrate RAR pan-antagonist was able to block the growth cone turning induced by atRA (Carter et al., 2015). Studies from our laboratory have provided preliminary evidence of the effects of synthetic RAR agonists (Nasser, 2017), but we currently only have limited evidence for a potential role for the invertebrate RAR in mediating the chemotropic effects of retinoids.

1.6 Specific aims of thesis

The goal of this thesis is to characterize the intracellular signalling cascades that mediate the effects of retinoic acid on growth cone guidance. This research employs cultured motorneurons isolated from the pond snail *Lymnaea stagnalis* as a model system. Two major questions are addressed in these studies.

Specific Aim 1: Can synthetic vertebrate RAR agonists mimic the effects of retinoic acid to induce growth cone turning?

In addition to the classical genomic effects of retinoids and their receptors, there is now increasing evidence for non-genomic effects of retinoic acid (Maghsoodi et al., 2008; Farrar et al., 2009; Rondina et al., 2016). Since the identification of *Lymnaea* RARs in the neurites and specifically in the growth cones of cultured neurons, a role for retinoid receptors in retinoic acid-mediated growth cone guidance has been proposed. However, we

currently only have limited evidence for a potential role for the RAR in mediating chemotropic effects of retinoids. Therefore, the present study aims to directly compare the chemotropic effects of retinoic acid with selective vertebrate RAR agonists. These studies will provide important information for the potential role of the RAR in mediating axon guidance.

Specific Aim 2: What are the downstream signalling pathways through which retinoic acid exerts its effects on growth cone guidance?

Retinoic acid-mediated growth cone guidance has been shown to require local protein synthesis and calcium influx (Farrar et al., 2009). The transduction mechanisms by which calcium might affect growth cone behaviours are not yet known. Rho GTPases are known effectors of calcium that may act as a link between calcium changes and growth cone cytoskeletal movement. Therefore, this study tests the hypothesis that retinoic acid mediates its effects on growth cone guidance via a second messenger cascade involving the Rho GTPases, Rac and Cdc42.

Chapter 2:

Materials and Methods

Animals

L. stagnalis were bred in the laboratory and kept in open air tanks containing aerated artificial pond water at room temperature. Water was supplemented with Instant Ocean Sea Salt at a concentration of 0.6 g/L. Animal nutrition consisted of Nutrafin Max Spirulina fish food and romaine lettuce, provided *ad libitum.*

Preparation of poly-L-lysine coated culture dishes

Cell culture dishes (Falcon Easy Grip Petri dishes, 35 x 10 mm) were prepared by drilling a hole (1 cm in diameter) through the bottom of the dish. A glass coverslip was then glued to the dish below the drilled hole. Dishes were sterilized with 70% ethanol (EtOH) and once dry, were coated overnight with poly-L-lysine (1 mg/mL Tris buffer). A glass pipette was used to remove the poly-L-lysine and the dishes were left to dry for 30 minutes. Dishes were then washed with sterile distilled water three times (15 minutes each) and left to dry. Poly-L-lysine-coated culture dishes were stored between 2 and 8°C and used within two weeks.

Preparation of Lymnaea conditioned medium

Lymnaea conditioned medium (CM) contains unidentified trophic factors that support neurite outgrowth *in vitro* (Wong et al., 1981). CM was prepared by dissecting 12 *Lymnaea* CNS from animals ranging in size from 20-30 mm. CNS were passed through a series of 18 washes in antibiotic saline (ABS; 225 μg/mL gentamicin), for a duration of 7-10 minutes each. Next, the 12 CNS were incubated in 7.5 mL of Defined Medium (DM) in Sigma-coted (Sigma-Aldrich) glass dishes (Pyrex, 60 x 15 mm). DM consists of 50% L-

15 (Leibowitz) media, Gentamicin (25 μg/mL), 4X salts (NaCl 40 mM, KCl 1.7 mM, $CaCl_2$ 4.1 mM, $MgCl_2$ 1.5 mM and HEPES 10 mM), Glutamine (60 mg/L), D-Glucose (6 mg/L), and autoclaved distilled water (24.6%); pH 7.9. Following an incubation period of four days, CNS were passed through an additional eight ABS washes and transferred to DM for two subsequent incubations (four days each). CM produced from the last two incubation periods were used for cell culture experiments. CM was checked for contamination prior to use, using an inverted microscope (Zeiss Axiovert 200) and discarded if compromised.

Cell culture procedures

All animals used to obtain neurons for cell culture ranged in size from 16 to 20 mm in length and all cell culture procedures were performed as described previously (Syed et al., 1990; Dmetrichuk et al., 2006). Under sterile conditions, individual CNS were removed from anaesthetized animals (25% Listerine® in saline). The isolated CNS were passed through a series of three, 10 minute ABS washes. Next, CNS were trypsinized (2 mg/mL DM; Sigma-Aldrich) for 19.5 to 21 minutes, followed by treatment with a trypsin inhibitor (2 mg/mL DM; Sigma-Aldrich) for 10 minutes. CNS were then pinned out in a dissection dish containing high osmolarity DM (800 μL of 1 M glucose in 30 mL DM) to minimize cell rupture during culture procedures. The outer connective tissue and inner sheath surrounding the left and right pedal ganglia were removed. Pedal A (PeA) ciliary motorneurons were individually isolated from the ganglia using a fire-polished glass pipette coated with Sigmacote (Sigma-Aldrich) to prevent cell adhesion. Suction was applied using a micrometer syringe to isolate individual cell bodies from the CNS. Individual PeA motorneurons were then plated in poly-L-lysine-coated dishes containing

2.5 mL of CM and 0.5 mL of DM. In DM alone, *Lymnaea* neurons do not normally extend neurites (Spencer et al., 1996), however, neurite regeneration has been demonstrated if cultured in CM (Spencer et al., 2000). To promote neurite outgrowth, atRA was added to the culture dishes following cell plating (10^{-7} M final bath concentration; Maden et al., 1998; Dmetrichuk et al., 2006). Neurite outgrowth was observed 16 to 18 hours following plating.

Figure 2.1: The CNS of *Lymnaea stagnalis*. *Lymnaea* CNS pinned out in a dissection dish containing antibiotic saline. Scale bar: 1 mm.

Chemicals

AtRA and 9-*cis* RA were obtained from Sigma-Aldrich. Retinoids were prepared in absolute EtOH and diluted using DM to a concentration of 10^{-5} M in the pipette (0.1% final EtOH concentration). Vehicle control experiments used a concentration of 0.1% EtOH in the pipette. The synthetic RAR agonists, EC23 (synthetic analog of atRA; Charton et al., 2009), and AM80 (a selective RARα agonist; Bernard et al., 1992) were obtained

from Tocris Bioscience. Synthetic retinoids were prepared in 100% dimethyl sulfoxide (DMSO) and diluted using DM to a concentration of 10^{-5} M in the pipette (0.1% final DMSO concentration). Vehicle control experiments used a concentration of 0.1% DMSO in the pipette.

NSC23766 (Sigma-Aldrich), an inhibitor of Rac, was prepared in sterile distilled water and diluted using DM to produce a final bath concentration of 10^{-4} M (Hou et al., 2014; Nasser, 2017). ML141 (Sigma-Aldrich), an inhibitor of Cdc42, was prepared in DMSO and diluted using DM to produce a final bath concentration of 10^{-5} M (Jiang et al., 2015) for experiments involving intact growth cones in response to atRA (0.1% final DMSO concentration). Due to solubility issues of ML141, for all experiments involving isolated growth cones and vehicle controls in ML141, ML141 was prepared in a 1:2 dilution of DMSO and Tween-80 (Johnson, 2013) and diluted using DM to produce a final bath concentration of 10^{-5} M (0.1% final DMSO concentration and Tween-80 at 0.2%). Vehicle control (for bath application) used DMSO and Tween-80 prepared in the exact same manner, but in the absence of ML141. All inhibitors were added to the bath at least 1 hour prior to growth cone turning assays.

Growth cone turning assays

All growth cone assays were conducted in a similar manner as previously described (Farrar et al., 2009; Carter et al., 2010; 2015). Individual growth cones were monitored, and images captured using an inverted microscope (Zeiss Axiovert 200) and Northern Eclipse imaging software. Only active growth cones exhibiting a steady trajectory were used for growth cone turning assays. Retinoic acid (atRA or 9-*cis* RA) or the synthetic

RAR agonists (EC23 or AM80) were focally applied to one side of the growth cone using a pressure pipette (Eppendorf-Femtojet) at a distance of 125 to 175 μM. Application pressure varied between 3-10 hPa depending on the size of the pipette and distance from the growth cone. A holding pressure of 1-2 hPa was used to prevent backflow into the pipette. Retinoids were placed in the pipette at a concentration of 10^{-5} M, but previous assays using similar approaches have shown that the concentration of a guidance cue reaching the growth cone is 100-1000 times less than that contained in the pipette (Lohof et al., 1992). Control experiments included use of the vehicle EtOH (0.1%) or DMSO (0.1%) in the pipette and were performed in the exact same manner. Control experiments using DM alone were not performed, as our laboratory has previously determined that application of DM does not produce growth cone turning (Dmetrichuk et al., 2006).

To examine the role of the Rho GTPases Rac and Cdc42 in retinoic acid-mediated growth cone turning, a pharmacological inhibitor of Rac (NSC23766) or Cdc42 (ML141) was bath applied at least 1 hour prior to growth cone turning assays. Retinoids (atRA or 9-*cis* RA) were then applied to growth cones that continued to actively grow in the presence of the inhibitor.

In order to examine the local response of growth cones in the absence of the soma, neurites were physically transected from the cell body using a glass electrode. After transection, growth cones were allowed approximately 15 minutes to recover from injury and monitored to ensure continued growth. Isolated growth cones that remained viable and were actively advancing were used for growth cone turning assays. A gradient of retinoic acid was applied to growth cones in a similar manner as that previously described for intact

growth cones. Transected neurites were monitored to ensure that the neurite did not re-establish a connection with the cell body.

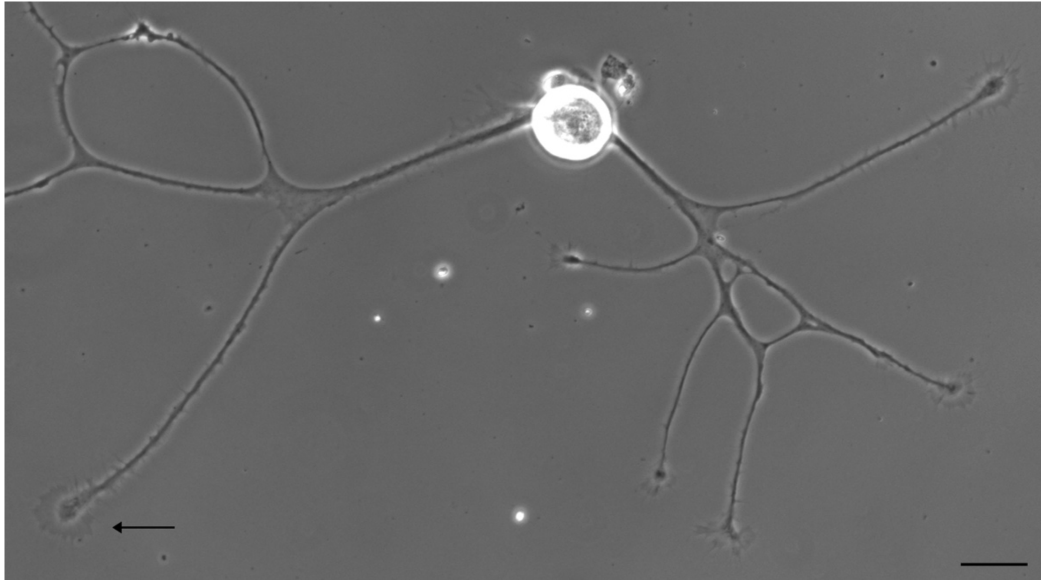


Figure 2.2: PeA neuronal outgrowth. Representative example of a PeA neuron in cell culture with extensive neurite outgrowth. Arrow indicates a growth cone at the tip of an extending neurite. Scale bar: 30 μM.

Growth cone measurements

The turning response of a growth cone was calculated by measuring the angle between the initial trajectory and the final trajectory of that growth cone following application of a retinoid (or vehicle). These angles were determined by an independent experimenter, blind to the conditions of the experiment. A positive turning angle indicates an attractive response, in which the growth cone turned towards the pipette containing the retinoid. A negative turning angle indicates a negative response, during which the growth cone turned away from the pipette. Growth cone trajectories were continually monitored throughout the experiments and images were captured using a Zeiss Axiovert 200 inverted microscope with Retiga Exi camera and Northern Eclipse software (Empix Imaging).

Immunostaining procedures

Following neuronal outgrowth, cultured cells were fixed for 20 minutes in 4% paraformaldehyde at room temperature. Samples were first washed for 10 minutes in PBS and permeabilized in 0.1% Triton-X/PBS for 30 minutes. Next, samples were blocked in 5% normal goat serum for 30 minutes and then incubated with the primary custom-made, affinity-purified, *Lym*RAR antibody (Pacific Immunology) diluted in blocking solution (1:50) at 4°C overnight. Negative control samples were incubated in blocking solution in the absence of the primary antibody. We have previously conducted preadsorption controls with this same primary antibody for immunostaining of *Lym*RAR in CNS neurons (Carter et al. 2015), and as such these were not repeated again here. The following day, all samples were washed in 0.1% Tween20/PBS (PBST), followed by incubation in the secondary antibody, Alexa Fluor-488 goat anti-rabbit (Invitrogen) for 2 hours at room temperature. The samples were then washed in PBST and counterstained with 4', 6-diamindino-2-phenylindole (DAPI) (Sigma) for 2 minutes (to observe nuclei). Samples were washed again in PBST followed by PBS and mounted in anti-fade Fluorosave mounting media (Sigma). Images were captured using an inverted microscope (Zeiss Axiovert 200) and Northern Eclipse imaging software.

Data and statistical analysis

Statistical analyses were preformed using SigmaStat software and graphs were generated using Graphpad Prism, Version 8.0 for Mac OS X. A one-way analysis of variance (ANOVA) was performed on growth cone data sets followed by a Tukey-Kramer *post hoc* test. For statistical analyses and comparisons of intact and isolated growth cone

data, a two-way ANOVA was performed, followed by a Tukey Kramer *post hoc* test. A Fisher's exact test was performed to determine the statistical significance of the observed frequency of growth cone turning during some experimental conditions. Data were expressed as mean ± standard error of mean (SEM) and were deemed significant when $p < 0.05$.

Chapter 3:

Results

3.1 The role of the RAR in axon guidance

In addition to the classical genomic effects of retinoids and their receptors, there is now increasing evidence for non-genomic effects of retinoic acid in many species (Maghsoodi et al., 2008; Farrar et al., 2009; Rondina et al., 2016). For example, studies from our laboratory have previously shown that both atRA and 9-*cis* RA can induce growth cone turning of regenerating cultured neurons in the absence of the cell body (and thus in the absence of gene transcription; Farrar et al., 2009). This turning response of isolated growth cones is dependent on local protein synthesis in the growth cone and requires calcium influx (Farrar et al., 2009), as is the case for many other traditional cues mediating axon guidance (Lin and Holt, 2007; Gomez and Zheng, 2006). It has previously been shown that a vertebrate RAR pan-antagonist was able to block the growth cone turning induced by atRA (Carter et al., 2015). However, the effects of synthetic RAR agonists have not fully been studied. As such, we currently have only limited evidence for a potential role for the invertebrate RAR in mediating chemotropic effects of retinoids. In this study, I have directly compared the chemotropic effects of atRA with two selective vertebrate RAR agonists. These include EC23 (synthetic analog of atRA and selective RAR pan-agonist; Charton et al., 2009), and AM80 (a selective RARα agonist; Bernard et al., 1992).

Lymnaea growth cones express LymRAR

Individual PeA motorneurons (which normally mediate ciliary behaviour during locomotion; Syed and Winlow, 1989) were isolated from the CNS and cultured overnight, during which time they regenerated neurites and exhibited actively motile growth cones within 18 hrs. I first confirmed that regenerating PeA motorneurons reliably exhibited

*Lym*RAR immunoreactivity. Using a custom-made, affinity purified antibody against *Lym*RAR, immunostaining confirmed that *Lym*RAR was expressed in the cell body of every cultured PeA neuron (Fig. 3.01A-B), regardless of whether it was actively regenerating or not (37/37 cells). Interestingly, as previously suggested (Carter et al., 2015), this *Lym*RAR was not evident in the nucleus (stained with DAPI; Fig. 3.01C). Of those neurons that displayed neurite outgrowth, *Lym*RAR immunoreactivity was also visualized in the majority of growth cones (28/33 growth cones; Fig. 3.01D-F). Interestingly, *Lym*RAR immunofluorescence was evident within the central domain of the growth cones, however, very weak (if any) RAR staining was visualized within the peripheral domain of the growth cone, consisting of the lamellipodia and filopodia (Fig. 3.01E). Control experiments which omitted the primary antibody were also performed, and regenerating PeA cells (in the absence of the primary antibody) showed no non-specific fluorescence (0/27 cell bodies and 0/23 growth cones; data not shown). These data support previous findings from our laboratory (Carter et al., 2015) that *Lym*RAR can be found in non-somatic compartments such as neurites and growth cones.

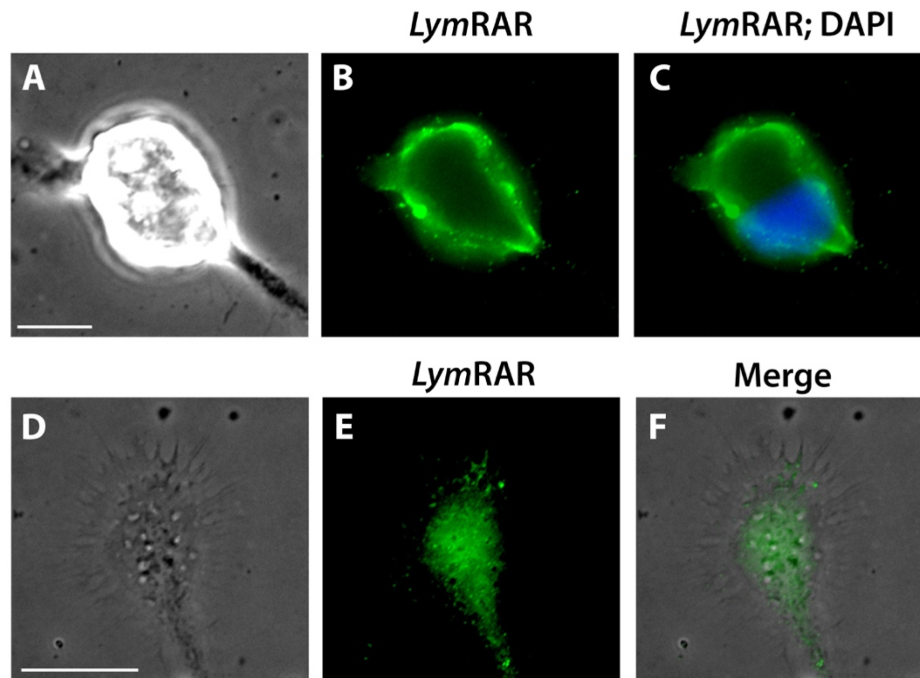


Figure 3.01: *Lym*RAR is located in the growth cones of regenerating cultured motorneurons. (**A**) Phase image of a PeA motorneuron. (**B**) Immunostaining of the same PeA motorneuron with *Lym*RAR antibody, showing mostly a non-nuclear distribution, as there is minimal overlap with the nuclear marker, DAPI, as shown in **C**. (**D**) Phase image of a regenerating growth cone of a PeA motorneuron. (**E**) The same growth cone showing immunostaining for *Lym*RAR contained mostly within the central domain of the growth cone. (**F**) Overlay of the *Lym*RAR expression with the phase image. Scale bars: 15 μM.

The synthetic retinoid EC23 mimics the chemotropic effects of atRA

Focal application of atRA to PeA growth cones produced attractive growth cone turning with a mean turning angle of 37.5º ± 3.9 (n = 10). A representative growth cone turning response towards atRA is shown in Figure 3.02A-B and each growth cone turning angle is represented in Figure 3.02C. For all data, a positive turning angle represents a turn towards the retinoid (or vehicle), whereas a negative turning angle is a turn away from the retinoid (or vehicle). I next determined whether the application of various synthetic RAR agonists could mimic this retinoic acid-induced growth cone turning. Indeed, PeA growth cones subjected to focal application of EC23 exhibited positive turning angles (24.6º ± 3.1; n = 7; Fig. 3.02D-F), similar to those induced by atRA. In contrast, growth cones failed to turn towards the focal source of AM80 and produced an overall mean turning angle of -3.1º ± 6.7 (n = 8; Fig. 3.02G-J). Finally, as expected, growth cones also showed no turning towards the vehicle (0.1% DMSO), exhibiting a mean turning angle of -4.5º ± 7.6 (n = 11; Fig. 3.02K-M).

Statistical analysis was performed using a one-way ANOVA ($F_{(3,32)} = 12.391$, $p < 0.001$) followed by a Tukey Kramer *post-hoc* test. Growth cone turning towards EC23 was significantly different from the vehicle ($p = 0.012$), but was not significantly different from that produced by atRA ($p = 0.491$; Fig. 3.03). Additionally, the turning angle produced by AM80 was not different than that elicited by the vehicle alone ($p = 0.998$), and was significantly different than that produced by atRA ($p < 0.001$; Fig. 3.03). These results therefore demonstrate the ability of the synthetic RAR agonist, EC23, to mimic growth cone turning induced by atRA.

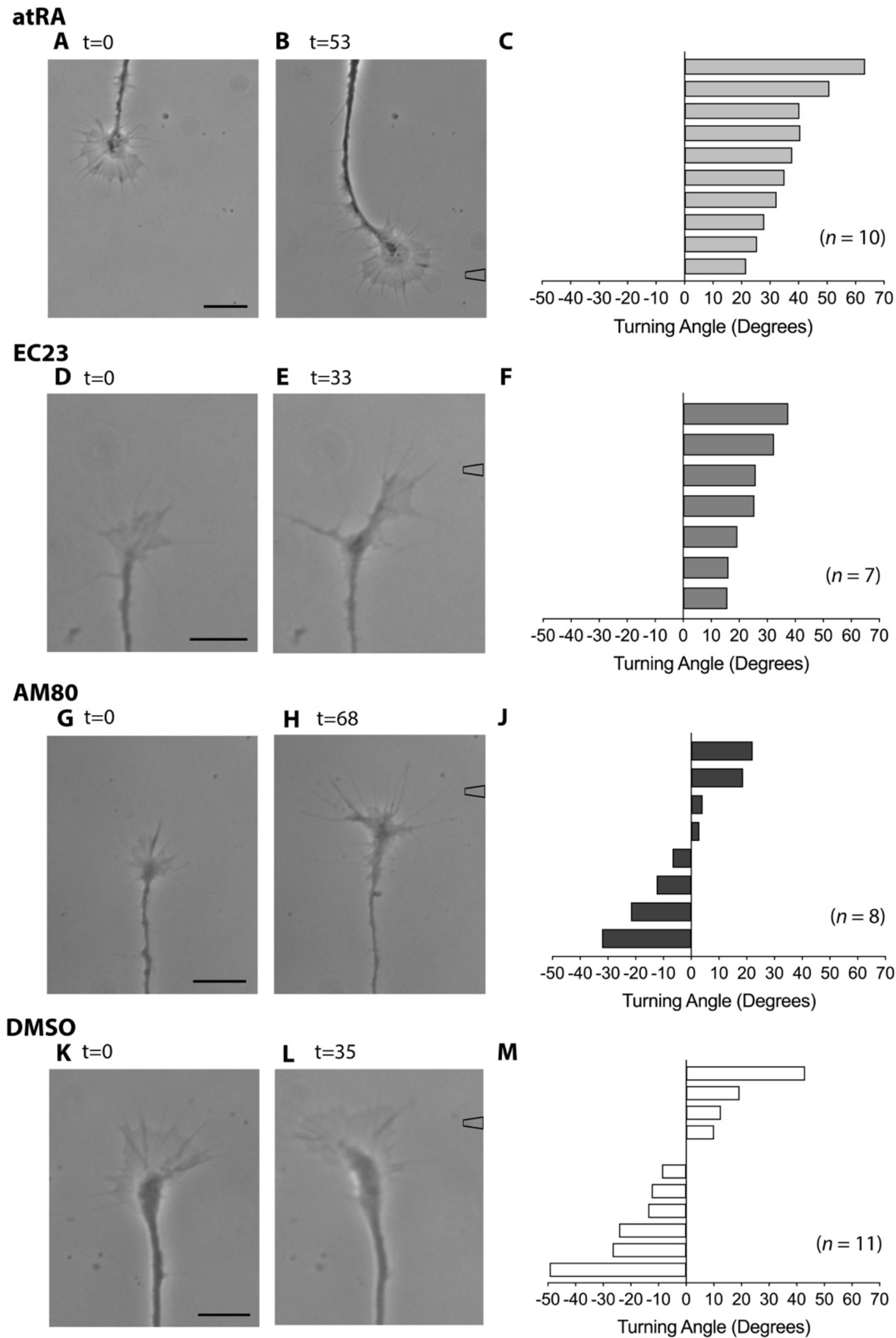


Figure 3.02: A synthetic retinoid can mimic the atRA-induced growth cone turning. Representative images of PeA growth cone responses to application of retinoids; atRA (**A-B**), EC23 (**D-E**), AM80 (**G-H**) and the vehicle DMSO (**K-L**). Growth cones turned towards atRA and EC23 but not AM80 (or DMSO). The approximate location of the pipette containing either retinoid or the vehicle is shown to the right of an image (**B, E, H, L**) in each data set. Graphs represent the maximum turning angle of individual growth cones to a gradient of atRA (**C**; *n* = 10), EC23 (**F**; *n* = 7), AM80 (**J**; *n* = 8), or DMSO (**M**; *n* = 11). Times (t) are given in minutes. Scale bars: 15 μM.

Collectively, these data (summarized in Figure 3.03), suggest that growth cone turning mediated by atRA is closely mimicked by the synthetic retinoid EC23, whereas AM80 had no obvious effect. Overall, these pharmacological analyses suggest that the efficacies of vertebrate RAR agonists in *Lymnaea* vary considerably. Despite this, I provide evidence that the non-chordate invertebrate RAR does indeed appear to be important for neurite guidance, as the RAR agonist EC23 (known to act at vertebrate RARs, but not RXRs; Gambone et al., 2002) was able to mimic the effects of retinoic acid.

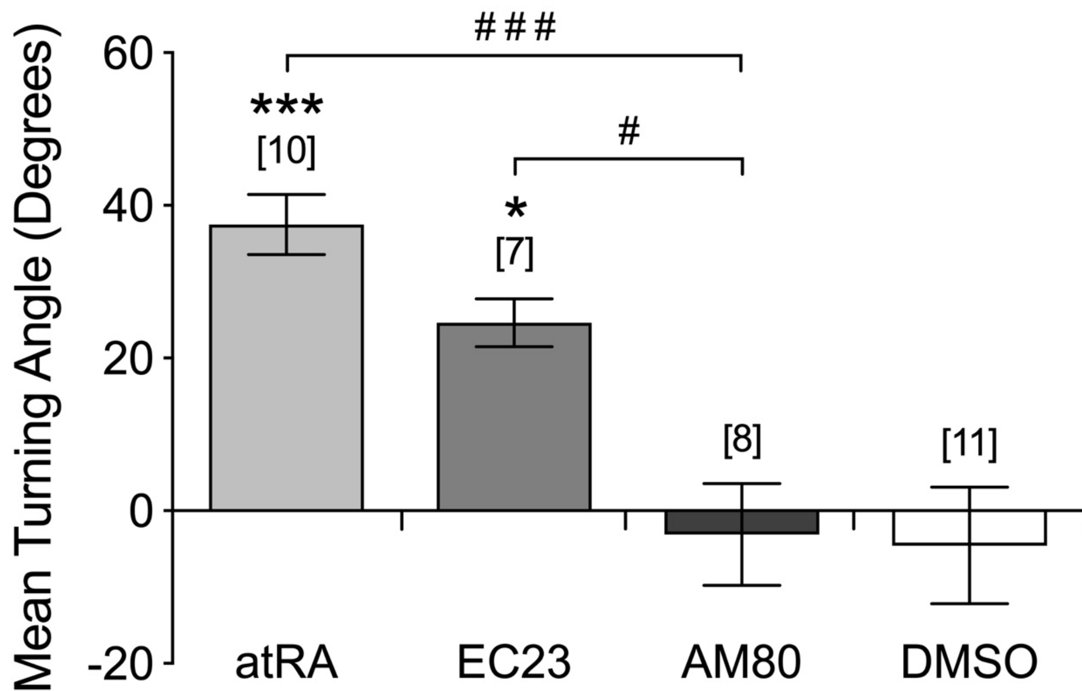


Figure 3.03: Summary of growth cone responses to all retinoids. Graph depicting the mean turning angles of growth cones responding to atRA, EC23, AM80, or DMSO. Data were expressed as the mean ± SEM and analyzed using a one-way ANOVA ($F_{(3,32)} = 12.391$, $p < 0.001$) followed by a Tukey Kramer *post-hoc* test. The mean turning angles produced by atRA (*** $p < 0.001$) and EC23 (* $p < 0.05$) were significantly greater than that elicited by DMSO. AM80 did not produce a significantly greater turning angle when compared to DMSO and the mean turning angle in response to AM80 was significantly reduced compared to that of both atRA and EC23 (### $p < 0.001$; # $p < 0.05$). *N* values for each group are provided in brackets.

3.2 The role of Rac in 9-*cis* RA-induced growth cone guidance

The presence of retinoic acid receptors in the growth cones, and the ability of a RAR agonist to mimic the turning response of retinoic acid, suggests a role for these neuritically localized receptors in the chemotropic response to retinoic acid. However, far less is known about the downstream signalling cascades activated by retinoic acid in this response. Previous studies have shown the involvement of the Rho GTPase, Rac, in growth cone turning towards atRA (Nasser, 2017). Specifically, in the presence of the Rac inhibitor, atRA induced growth cone collapse and retraction (Nasser, 2017). Therefore, this next study aimed to examine the role of the Rho GTPase, Rac, in 9-*cis* RA-mediated growth cone guidance.

Growth cone turning mediated by 9-cis RA is blocked by the inhibition of Rac

I first confirmed that growth cones of cultured PeA motorneurons reliably turned towards a local gradient of 9-*cis* RA to ensure the repeatability of previous findings. Consistent with previous studies (Dmetrichuk et al., 2008; Carter et al., 2010), focal application of 9-*cis* RA (10^{-5} M in pipette) produced attractive growth cone turning of regenerating neurites (mean turning angle: 38.5° ± 7.7; n = 10), and a representative example is shown in Figure 3.04A-B. In contrast, application of the vehicle alone (as a control) failed to induce growth cone turning towards the pipette, producing a mean turning angle of -5.2° ± 4.5 (n = 8; Fig. 3.04D-E). Graphical representations of all individual growth cone turning angles in response to either 9-*cis* RA or the vehicle are illustrated in Figures 3.04C and 3.04F, respectively.

The growth cone turning responses towards 9-*cis* RA were next determined in the presence of a Rac inhibitor, NSC23766 (a selective inhibitor of Rac1-GEF interaction; 10^{-4} M final bath concentration; Nasser, 2017). The attractive response normally exhibited by a gradient of 9-*cis* RA was completely abolished in the presence of the Rac inhibitor (mean turning angle: -18.8° ± 5.5; $n = 8$), and 7 of 8 growth cones turned away from the source of 9-*cis* RA. A representative growth cone turning response to 9-*cis* RA in the presence of the Rac inhibitor is shown in Figure 3.04G-H, and individual growth cone turning angles are represented in Figure 3.04J.

A summary of the mean growth cone responses to 9-*cis* RA in the presence and absence of NSC23766, and vehicle control, are shown in Figure 3.05. Statistical analysis was performed using a one-way ANOVA ($F_{(2,23)} = 23.226$, $p < 0.001$) followed by a Tukey Kramer *post-hoc* test. The mean turning angle produced by 9-*cis* RA in the presence of the Rac inhibitor was significantly reduced from the turning angle elicited in the absence of the Rac inhibitor ($p < 0.001$). Additionally, this mean turning angle in the presence of the Rac inhibitor was not significantly different from that produced by the vehicle alone ($p = 0.330$). A Fisher's exact test further confirmed that there was no significant association between the observed frequency of growth cone turning away in response to 9-*cis* RA in the presence of the Rac inhibitor versus in the presence of the vehicle alone ($p = 0.282$).

Overall, these findings indicate that Rac may have an important role in mediating growth cone turning towards 9-*cis* RA.

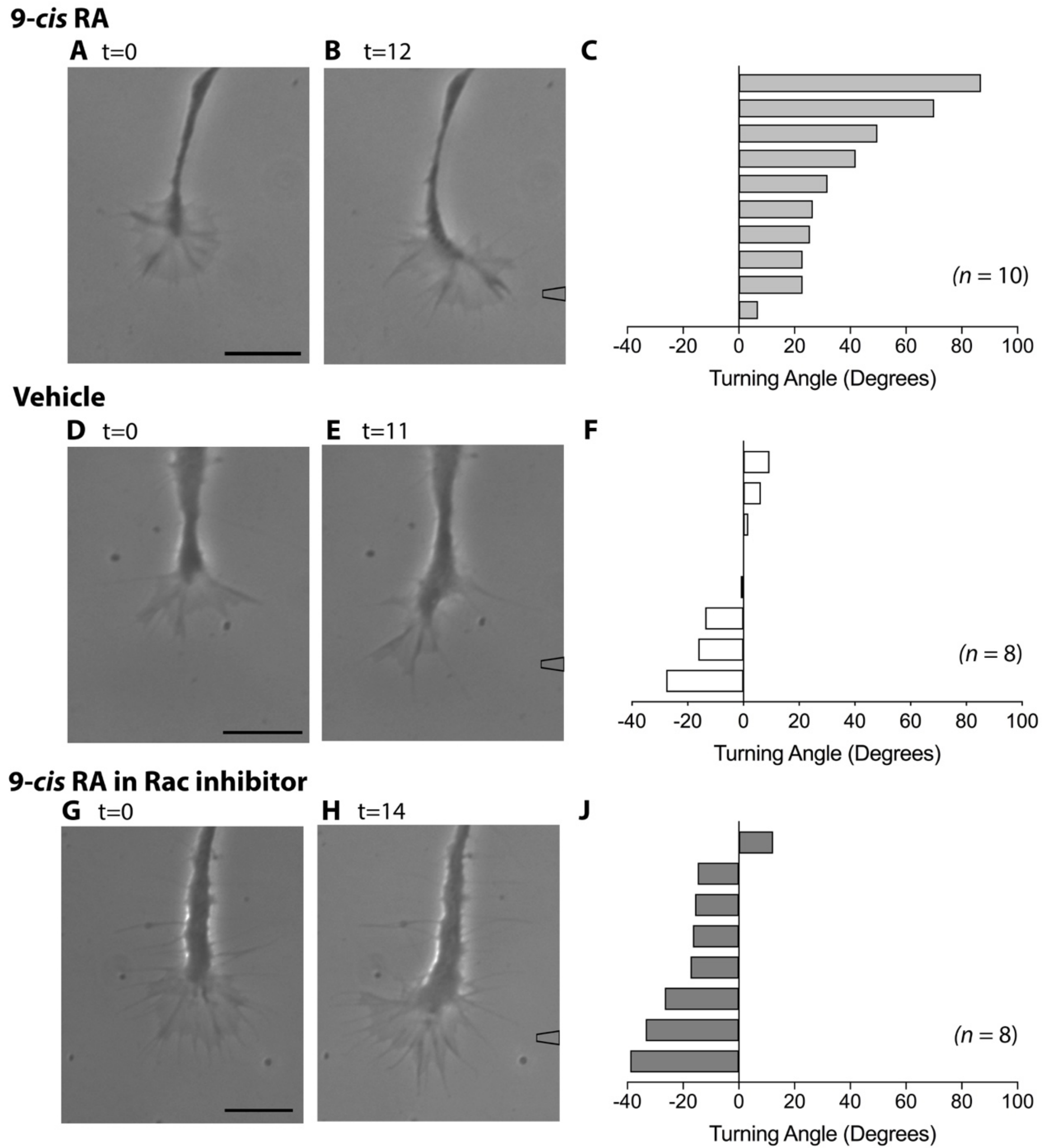


Figure 3.04: Growth cone turning mediated by 9-*cis* RA is blocked by the inhibition of Rac. Representative images depicting the turning response of PeA growth cones to 9-*cis* RA in the absence (**A-B**) or presence (**G-H**) of the Rac inhibitor. Growth cone turning responses to the vehicle are also shown (**D-E**). The approximate location of the pipette containing either 9-*cis* RA or vehicle is shown to the right of an image (**B, E, H**) in each data set. Graphs represent the maximum turning angle of individual growth cones in each condition (**C**; *n* = 10), (**F**; *n* = 8), and (**J**; *n* = 8). Times (t) are given in minutes. Scale bars: 15 μM.

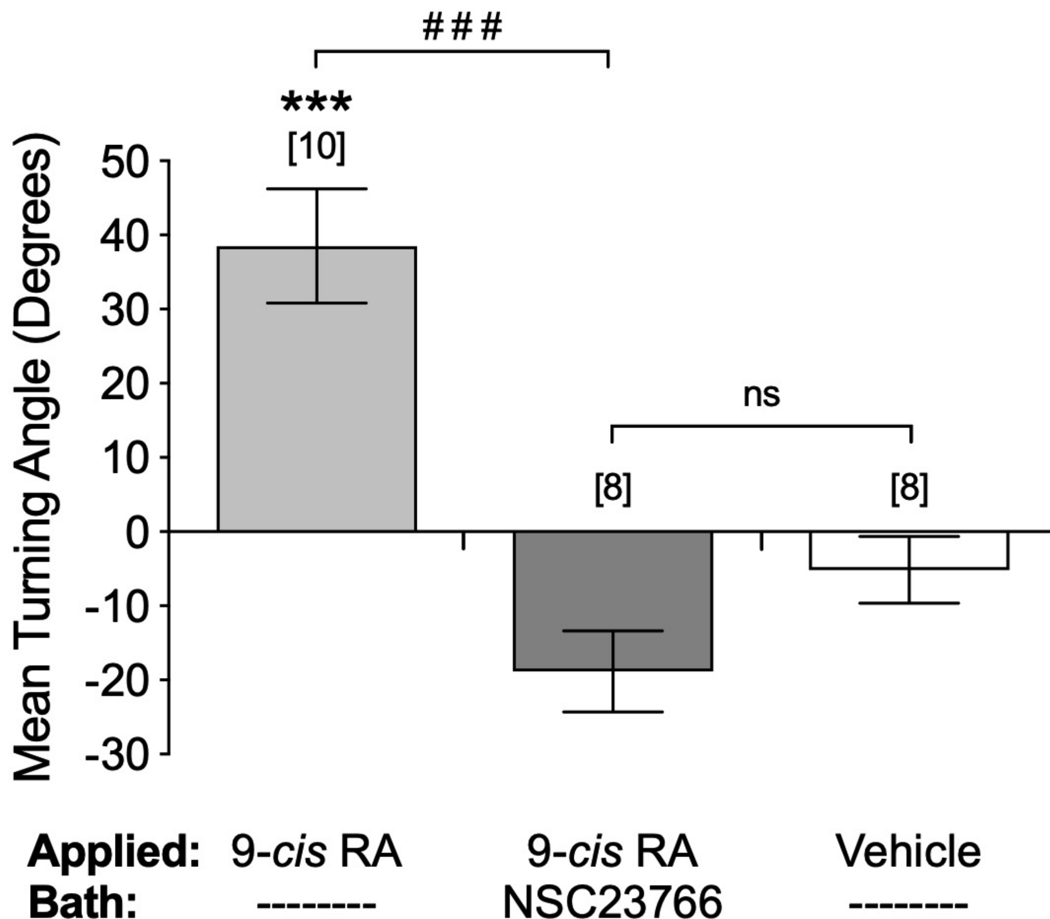


Figure 3.05: Growth cones fail to turn towards 9-*cis* RA in the presence of the Rac inhibitor. Graph depicting the mean turning angles of growth cones in response to 9-*cis* RA in the presence and absence of the Rac inhibitor (NSC23766), as well as the turning response to the vehicle control. Data were expressed as the mean ± SEM and analyzed using a one-way ANOVA ($F_{(2,23)} = 23.226$, $p < 0.001$) followed by a Tukey Kramer *post-hoc* test. The mean turning angle produced by 9-*cis* RA was significantly greater than that elicited by the vehicle (***$p < 0.001$). The mean turning angle produced by 9-*cis* RA in the presence of the Rac inhibitor was significantly reduced from the turning angle elicited in the absence of the Rac inhibitor (###$p < 0.001$) and was not significantly different from the turning angle produced by the vehicle (ns: not significant). *N* values for each group are provided in brackets.

The Rac inhibitor does not inhibit 9-cis RA-induced growth cone turning of isolated growth cones

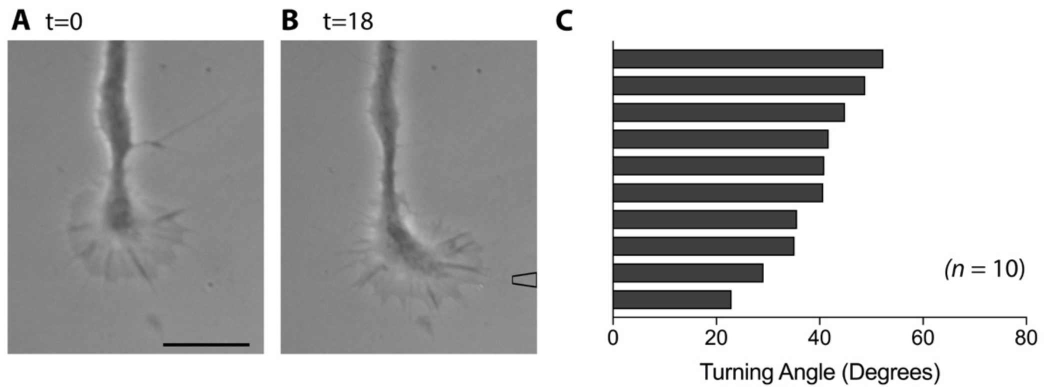
Growth cones that are isolated from the cell body have been shown to maintain the ability to respond to guidance cues. It has previously been shown that *Lymnaea* growth cones and neurites that are severed from the soma continue to respond and turn towards a gradient of retinoic acid. Furthermore, this turning response of isolated growth cones occurs in the absence of gene transcription, and requires local protein synthesis (Farrar et al., 2009). Accordingly, my next aim was to use isolated growth cones to examine the involvement of Rac in localized signalling mechanisms within the growth cone that are independent of communication with the cell body.

Growth cones were physically isolated from the cell body and then allowed time to recover from injury. Isolated growth cones that remained viable and were actively advancing were exposed to a gradient of 9-*cis* RA. I first demonstrated that growth cones isolated from their cell bodies retained the ability to turn towards a gradient of 9-*cis* RA (10^{-5} M in pipette) with a mean turning angle similar to those of intact growth cones (mean turning angle: 39.2° ± 5.6; $n = 10$; Fig. 3.06A-B). I was next interested in examining the effects of the Rac inhibitor (NSC23766) on isolated growth cone turning towards 9-*cis* RA. Interestingly, treatment of isolated growth cones with the Rac inhibitor had no effect on growth cone turning towards 9-*cis* RA. That is, isolated growth cones in the presence of the Rac inhibitor continued to turn towards the focally applied 9-*cis* RA with a mean turning angle of 42.3° ± 5.9 ($n = 9$; Fig. 3.06D-E). The marked attractive growth cone turning towards 9-*cis* RA is clearly depicted by the graphs showing the maximum turning

angles of individual, isolated growth cones in the absence or presence of the Rac inhibitor (Fig. 3.06C and 3.06F, respectively).

A summary graph comparing the mean turning angles of intact or isolated growth cones in response to 9-*cis* RA, in the presence or absence of NSC23766 is shown in Figure 3.07. A two-way ANOVA revealed a significant interaction effect between the bath treatment (Rac inhibitor) and the condition (intact or isolated) of the growth cone ($F_{(1,33)}$ = 26.740, $p < 0.001$). A Tukey Kramer *post-hoc* test revealed a significant reduction in the mean turning response of intact growth cones to 9-*cis* RA in the presence of the Rac inhibitor ($p < 0.001$). In contrast, the response of isolated growth cones to 9-*cis* RA was not significantly different in the presence of the Rac inhibitor ($p = 0.706$).

Taken together, these studies indicate that the turning response of intact growth cones towards 9-*cis* RA was blocked by the inhibition of Rac. One interesting finding in this study is that the Rac inhibitor did not have the same ability to inhibit isolated growth cone turning towards 9-*cis* RA. These results therefore demonstrate that involvement of Rac (or effectiveness of inhibitor) in growth cone turning varies, depending on whether the growth cones have maintained communication with the cell body.



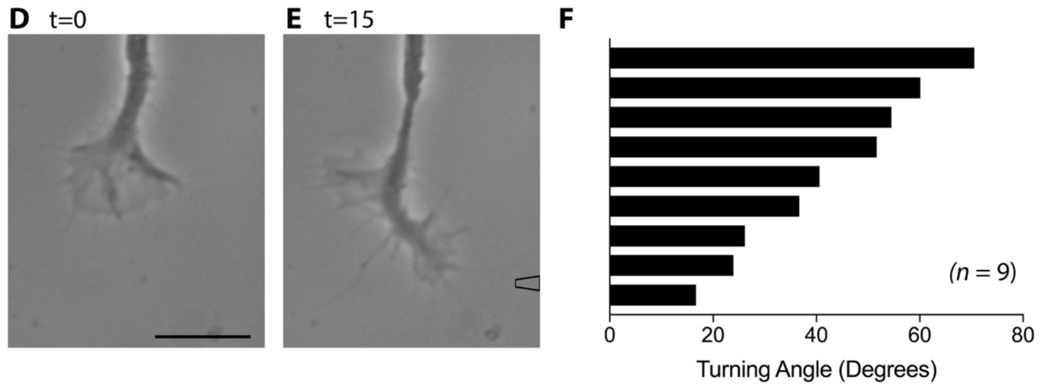


Figure 3.06: Isolated growth cones continue to turn towards 9-*cis* RA in the presence of the Rac inhibitor. Representative images depicting the turning response of isolated PeA growth cones to 9-*cis* RA in the absence (**A-B**) or presence (**D-E**) of the Rac inhibitor. The approximate location of the pipette containing 9-*cis* RA is shown to the right of an image (**B, E**) in each data set. Graphs represent the maximum turning angle of individual growth cones in each condition (**C**; n = 10) and (**F**; n = 9). Times (t) are given in minutes. Scale bars: 15 µM.

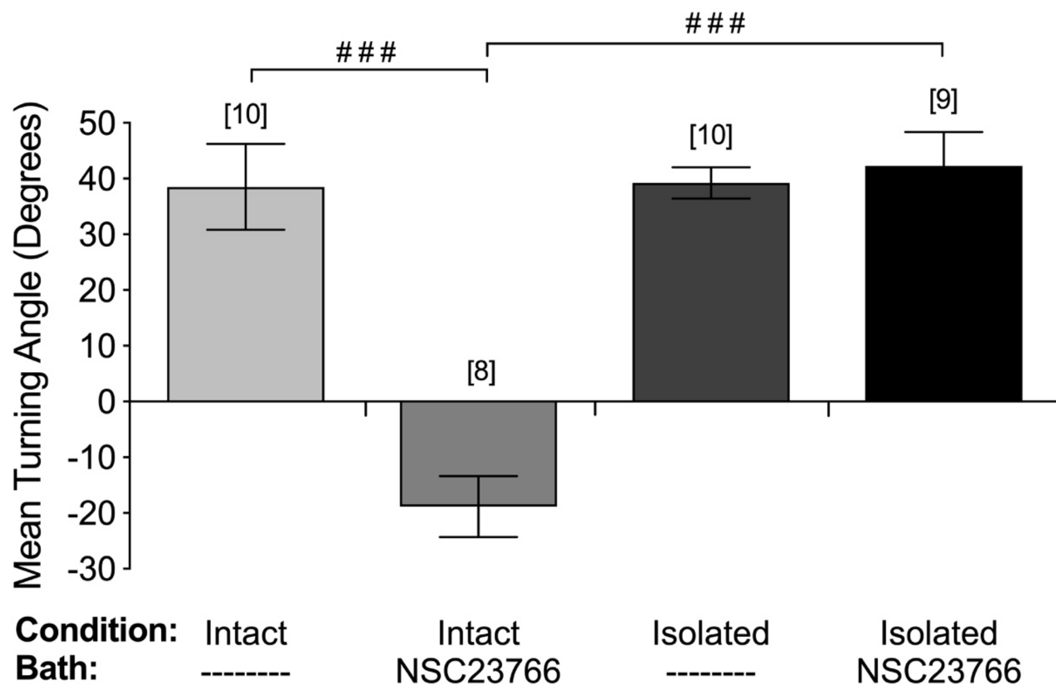


Figure 3.07: The Rac inhibitor does not inhibit isolated growth cone turning towards 9-*cis* RA. Graph depicting the mean turning angles of intact or isolated growth cones in response to 9-*cis* RA, in the presence or absence of the Rac inhibitor (NSC23766). Data were expressed as the mean ± SEM and analyzed using a two-way ANOVA ($F_{(1,33)} = 26.740$, $p < 0.001$) followed by a Tukey Kramer *post-hoc* test. NSC23766 inhibited growth cone turning towards 9-*cis* RA of intact, but not isolated growth cones (###$p < 0.001$). *N* values for each group are provided in brackets.

3.3 The role of Cdc42 in atRA-induced growth cone guidance

In view of the essential role for the Rho GTPases, Rac and Cdc42, in positively regulating growth cone extension to other specific extracellular cues, I next sought to investigate the requirement for Cdc42 in retinoic acid-induced growth cone turning.

The inhibition of Cdc42 induces a switch in growth cone responsiveness to atRA

A local gradient of atRA was first applied to regenerating neurites of cultured motorneurons. Due to the capacity of the retinoid isomers to act in a similar manner, and the involvement of Rac in growth cone turning towards both isomers, only atRA was exogenously applied to growth cones in the following experiments. Similar to the effects of 9-*cis* RA, atRA (10^{-5} M in pipette) also produced attractive growth cone turning with a mean turning angle of 37.5° ± 3.9 (n = 10). A representative example of a growth cone responding to atRA is shown in Figure 3.08A-B and the maximum turning angles of individual growth cones are summarized in Figure 3.08C.

In contrast to the Rac inhibitor, the Cdc42 inhibitor used in these studies was dissolved in DMSO. Thus, prior to testing the turning responses in the Cdc42 inhibitor, I first confirmed that the growth cones response to atRA would not change in the presence of the vehicle (DMSO) in the bath. In the presence of the vehicle alone (0.1% DMSO), growth cones continued to turn towards the focally applied atRA (mean turning angle: 38.5° ± 5.3; n = 10; Fig. 3.08D-E). The growth cone turning responses towards atRA were next examined in the presence of the Cdc42 inhibitor, ML141 (10^{-5} M; final bath concentration). In the presence of the Cdc42 inhibitor, growth cones failed to turn towards atRA exhibiting a mean turning angle of -41.7° ± 5.6 (n = 10). The inhibition of growth

cone turning towards atRA is clearly depicted by the representative images shown in Figure 3.08G-H. Turning angles of individual growth cone responses to atRA in the presence of the Cdc42 inhibitor or in the presence of the vehicle are shown in Figures 3.08J and 3.08F, respectively.

Interestingly, in the above studies the inhibition of Cdc42 not only prevented growth cone turning towards atRA, but also appeared to convert atRA-induced attraction to repulsion. A switch in response from attraction to repulsion, rather than inhibition alone, may enable growth cones to respond differently to the same guidance molecule at different stages during nervous system development. It is however possible that this switch in responsiveness is not cue specific, but rather due to pressure artifact from the pipette application of solution. That is, the Cdc42 inhibitor may have increased growth cone susceptibility to the pressure of chemical application. In order to determine whether the Cdc42 inhibitor increased growth cone susceptibility to pressure, further experiments were performed in which the vehicle alone was applied to growth cones in the presence of the Cdc42 inhibitor. Growth cones did not turn towards the source of the vehicle, as expected, and a slightly negative turning angle was observed in response to the vehicle in the presence of the Cdc42 inhibitor (mean turning angle: -3.1° ± 3.3; n =10; Fig. 3.08K-L). The individual turning angles in response to the vehicle in the presence of the Cdc42 inhibitor are summarized in Figure 3.08M. These results suggest that growth cone repulsion in the presence of the Cdc42 inhibitor is in fact due to application of atRA and not due to the pressure of chemical application.

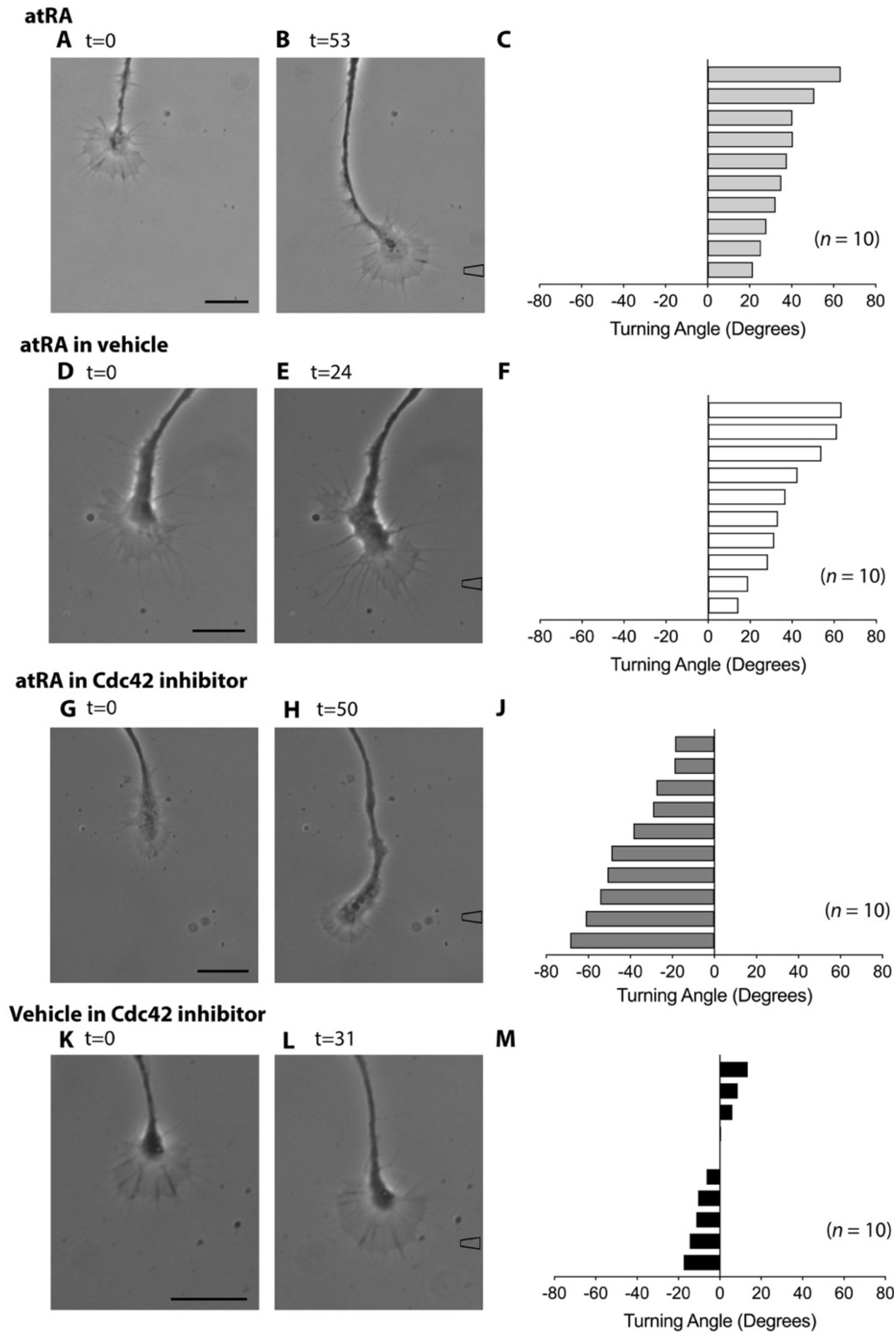


Figure 3.08: Inhibiting Cdc42 switches the atRA-induced growth cone response from attraction to repulsion. Application of atRA induced growth cone turning (**A-B**), which was maintained in the presence of DMSO in the bath (**D-E**). However, in the presence of the Cdc42 inhibitor, growth cones failed to turn towards atRA (**G-H**). Vehicle applied in presence of the Cdc42 inhibitor did not induce any consistent turning responses (**K-L**). The approximate location of the pipette containing either atRA or vehicle is shown to the right of an image (**B, E, H, L**) in each data set. Graphs represent the maximum turning angle of individual growth cones in each condition (**C**; n = 10), (**F**; n = 10), (**J**; n = 10) and (**M**; n = 10). Times (t) are given in minutes. Scale bars: 15 μM.

The mean turning angles in each condition are summarized in Figure 3.09. A one-way ANOVA revealed a significant effect ($F_{(3,36)} = 68.215$, $p < 0.001$), and a Tukey Kramer *post-hoc* test showed that there was a significant reduction in positive growth cone turning towards atRA in the presence of the Cdc42 inhibitor, as compared to in its absence ($p < 0.001$). Growth cone responses to atRA in the presence of the Cdc42 inhibitor significantly changed from atRA alone (in the presence of vehicle only; $p < 0.001$). Also, in the Cdc42 inhibitor a significantly greater negative turning angle was observed in response to atRA than in response to vehicle application alone ($p < 0.001$). Overall, these data indicate that inhibiting Cdc42 can induce a switch in growth cone responsiveness to atRA from attraction to repulsion.

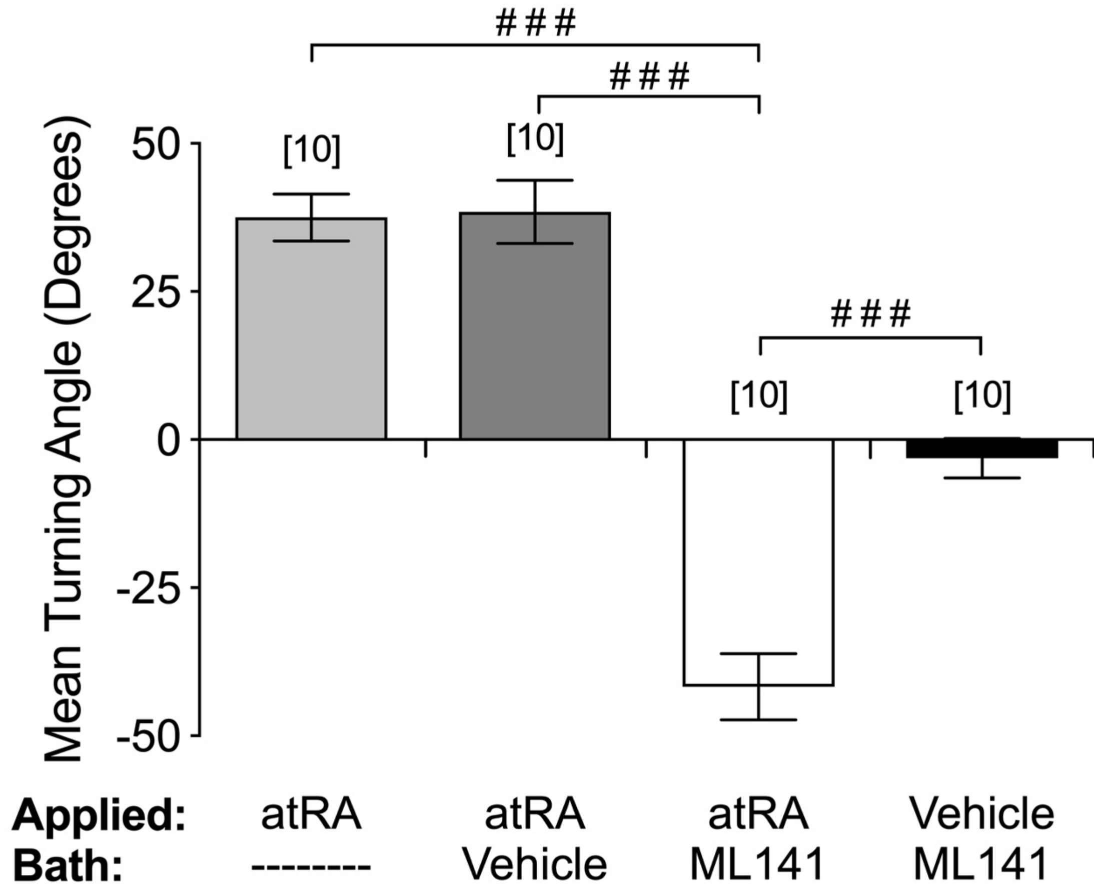


Figure 3.09: Growth cones turn away from atRA in the presence of the Cdc42 inhibitor. Graph depicting the mean turning angles of growth cones in response to atRA (or vehicle) in the presence or absence of the Cdc42 inhibitor (ML141). Data were expressed as the mean ± SEM and analyzed using a one-way ANOVA ($F_{(3,36)} = 68.2175$ $p < 0.001$) followed by a Tukey Kramer *post-hoc* test. In the presence of the Cdc42 inhibitor, the mean turning angle in response to atRA was significantly more negative (repulsive) than in all other experimental conditions (###$p < 0.001$). *N* values for each group are provided in brackets.

The Cdc42 inhibitor blocks atRA-induced growth cone turning of isolated growth cones

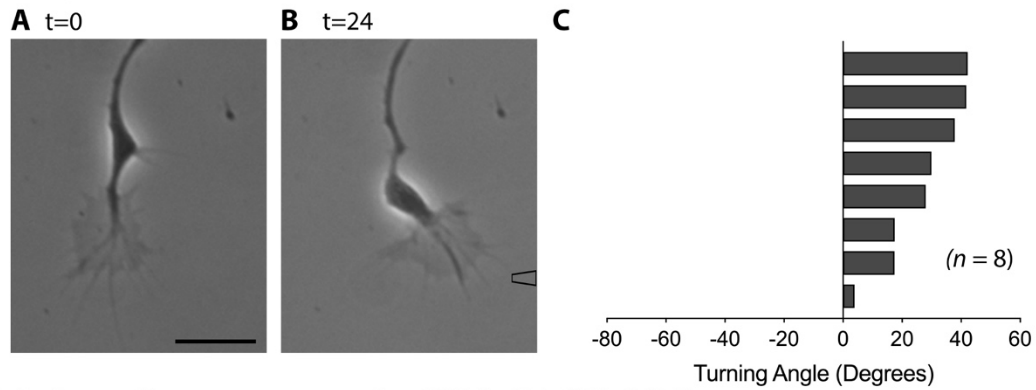
In the present study, I found that the involvement of Rac differed depending on whether growth cones maintained communication with the cell body. Therefore, I was next interested in examining if this was also true for the involvement of Cdc42 in atRA-mediated growth cone turning. Testing the ability of isolated growth cones to respond in the presence of ML141 will provide information on the requirement of Cdc42 for local signals within the growth cone.

The turning responses of isolated growth cones to atRA (10^{-5} M in pipette) were tested in the presence of the Cdc42 inhibitor (or the vehicle alone). In the presence of the Cdc42 inhibitor, isolated growth cones failed to initiate a turn towards the focally applied atRA (mean turning angle: -26.0° ± 6.3; $n = 10$) and a representative example is shown in Figure 3.10D-E. However, in the presence of the vehicle, isolated growth cones continued to turn towards the focally applied atRA with a mean turning angle of 27.3° ± 4.8 ($n = 8$; Fig. 3.10A-B). Turning angles of individual isolated growth cones to atRA, in the presence of the Cdc42 inhibitor or the vehicle are shown in Figures 3.10F and 3.10C, respectively. Further experiments were performed in which the vehicle alone was applied to isolated growth cones in the presence of the Cdc42 inhibitor. The purpose of these experiments was to determine whether the inhibition of Cdc42 induced a switch in growth cone responsiveness specifically to atRA, or just induced increased sensitivity to pressure application of any chemical (causing them to turn away from the applied chemical). In response to application of the vehicle instead of atRA, growth cones failed to turn towards the vehicle (mean turning angle: -9.8° ± 7.4; $n = 10$; Fig. 3.10G-H), but also did not produce a mean turning angle that was significantly different from isolated growth cone responses

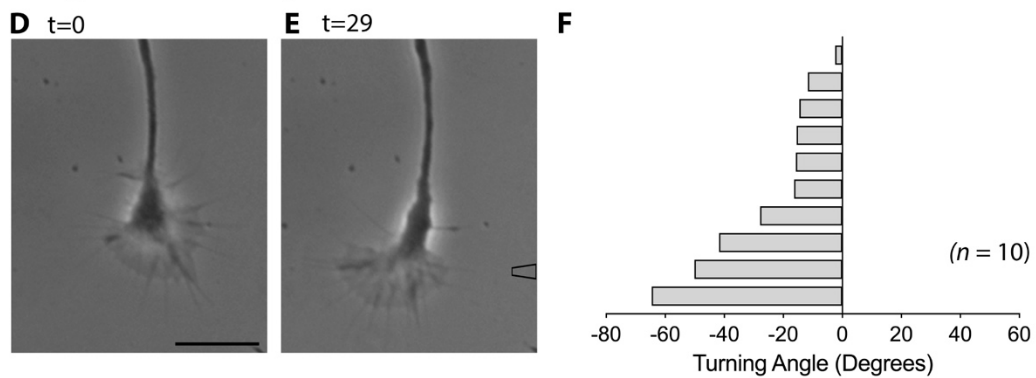
to atRA in the Cdc42 inhibitor ($p = 0.178$). A Fisher's exact test further confirmed that there was no significant association between the observed frequency of growth cone turning away in response to atRA in the presence of the Cdc42 inhibitor versus in response to the vehicle in the Cdc42 inhibitor; $p = 0.087$). The individual turning angles of isolated growth cones in response to the vehicle alone in the presence of the Cdc42 inhibitor are shown in Figure 3.10J.

The turning angles of isolated growth cones are summarized (Fig. 3.11A) and shown alongside those of intact growth cones for comparison (Fig. 3.11B). A one-way ANOVA on isolated growth cone data revealed a significant effect ($F_{(2,25)} = 16.610$, $p < 0.001$) and a Tukey Kramer *post hoc* analysis confirmed that the average attractive turning angles to atRA were significantly reduced in the presence of ML141, compared to in its absence (vehicle alone $p < 0.001$). In contrast to intact growth cones which showed a switch in responsiveness to atRA in the presence of the Cdc42 inhibitor, the turning response of isolated growth cones to atRA in the presence of the Cdc42 inhibitor was not significantly different than the turning response to the vehicle in the presence of the Cdc42 inhibitor ($p = 0.178$). Overall, these data suggest that inhibition of Cdc42 blocks growth cone turning regardless of whether the growth cone is intact or isolated.

Isolated growth cone response to atRA in vehicle



Isolated growth cone response to atRA in Cdc42 inhibitor



Isolated growth cone response to vehicle in Cdc42 inhibitor

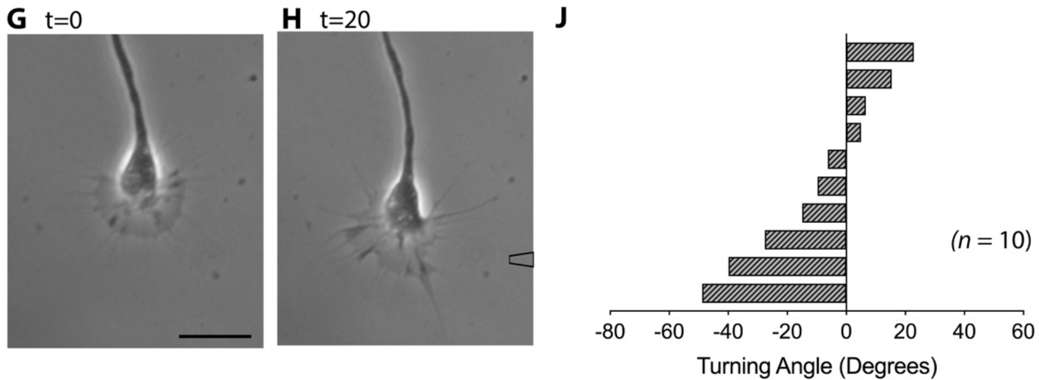


Figure 3.10: Isolated growth cones fail to turn towards atRA in the presence of the Cdc42 inhibitor. Representative images depicting the turning response of isolated PeA growth cones to atRA in the presence of the vehicle (**A-B**) or presence of the Cdc42 inhibitor (**D-E**). The isolated growth cone turning response to focal application of the vehicle in the presence of the Cdc42 inhibitor is also shown (**G-H**). The approximate location of the pipette containing either atRA or vehicle is shown to the right of an image (**B, E, H**) in each data set. Graphs represent the maximum turning angle of individual growth cones in each condition (**C**; *n* = 8), (**F**; *n* = 10) and (**J**; *n*=10). Times (t) are given in minutes. Scale bars: 15 μM.

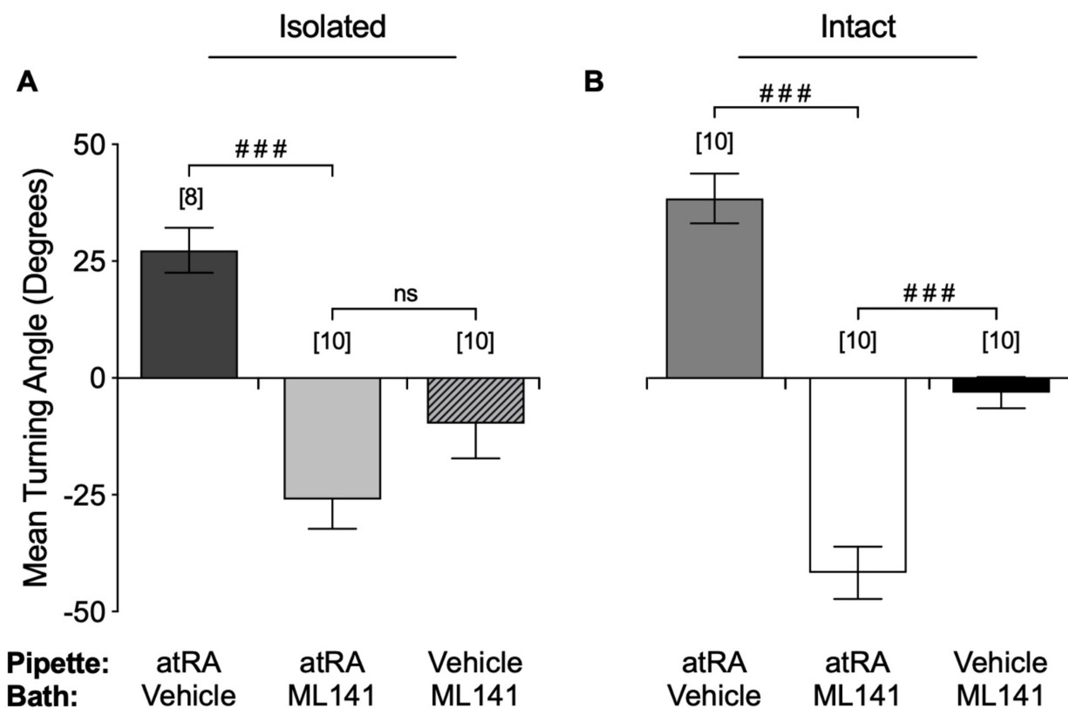


Figure 3.11: The Cdc42 inhibitor blocks atRA-induced growth cone turning of isolated growth cones. (**A**) Graph depicting the mean turning angles of isolated growth cones in each condition. Data were expressed as the mean ± SEM and analyzed using a one-way ANOVA ($F_{(12,25)} = 16.610$, $p < 0.001$) followed by a Tukey Kramer *post-hoc* test. The mean turning angle of isolated growth cones to atRA is significantly different in the presence of the Cdc42 inhibitor (ML141) as compared to in the presence of the vehicle (###$p < 0.001$). The mean turning angle produced by atRA in the presence of the Cdc42 inhibitor was not significantly different than the mean tuning angle produced by the vehicle in the presence of the Cdc42 inhibitor (ns: not significant). (**B**) Data for intact growth cones (shown previously in Fig. 3.09) are shown alongside for comparison (###$p < 0.001$). *N* values for each growth are provided in brackets.

In summary, I have provided evidence for a role of Rho GTPases in retinoid-induced growth cone guidance. I have shown that the inhibition of Rac or Cdc42 prevents growth cone turning towards retinoic acid. Interestingly however, the inhibition of Rac did not inhibit growth cone turning of isolated growth cones, suggesting its involvement might depend on whether the growth cones have maintained communication with the cell body. Moreover, the Cdc42 inhibitor not only blocked growth cone turning towards retinoic acid, but also induced a switch in growth cone responsiveness from chemoattraction to chemorepulsion in intact growth cones. These results therefore demonstrate that the signal transduction pathways underlying retinoic acid-mediated growth cone guidance involve the Rho GTPases, Rac and Cdc42.

Chapter 4:

Discussion and Conclusions

4.1 The potential role of *Lym*RAR in retinoic acid-induced growth cone turning

In this study, I first investigated the role of *Lym*RAR in potentially mediating the chemotropic effects of retinoic acid. Together with previous studies (Carter et al., 2015), this study now provides further evidence for a role of *Lym*RAR in mediating growth cone turning behaviour in actively growing neurites.

Retinoids are known to exert chemotropic effects in both invertebrate and vertebrate neurons. For example, atRA was first shown to attract neurites from cultured neural tube cells of the chick (Maden et al., 1998), as well as direct outgrowth from cultured newt spinal cord explants (Dmetrichuk et al., 2005). Interestingly, when retinoic acid is absent from quail embryos, motoneuron outgrowth from the neural tube is disrupted and misguided outgrowth occurs (Wilson et al., 2003), supporting a chemotropic role for atRA *in vivo*. The chemotropic effects of atRA in both newt spinal cord (Dmetrichuk et al., 2005) as well as cultured *Xenopus* neurons (Rand et al., 2017), were blocked by selective RAR antagonists. These previous findings suggest that the RAR can mediate the chemotropic effects of atRA in vertebrate neurons. This notion is now also supported for invertebrate neurons, as I show here that a selective vertebrate RAR agonist (EC23) can mimic atRA-induced growth cone turning (though the selective RARα agonist AM80 failed to do so).

I have demonstrated here that the synthetic retinoid EC23, known to act at vertebrate RARs, but not RXRs (Gambone et al., 2002), can closely mimic the effects of retinoic acid in an invertebrate protostome species. EC23 has previously been shown to exert similar effects to atRA in vertebrates, including the ability to induce neural differentiation (Christie et al., 2008; Maltman et al., 2009). Indeed, EC23 mimics atRA in

inducing differentiation of motoneurons from human embryonic stem cells (Christie et al., 2010) as well as differentiation of neurons from pluripotent human embryonal carcinoma cell lines (Clemens et al., 2013).

On the other hand, the synthetic agonist AM80 was not found to exert any effects on *Lymnaea* growth cones. Indeed, AM80 has previously been found to exert different biological activities compared to atRA, and has thus been proposed to act through different, or diverging signalling pathways. AM80 is considered an RAR agonist that exhibits selectivity for the vertebrate RARα, rather than the β or γ sub-type. This also differs from atRA which exhibits minimal selectivity across vertebrate RAR sub-types (Allenby et al., 1994). It should be noted that there have also been examples where AM80 exerts similar actions to atRA (Jetten et al., 1987; Jimi et al., 2007), and exhibits greater potency than atRA in inducing neuronal differentiation (Shiohira et al., 2010). In this study however, I found that AM80 was not able to mimic the effects of atRA in inducing growth cone turning. This study thus suggests that either the *Lym*RAR is not similar enough to the vertebrate RARα sub-type, or that far higher concentrations, above what I tested here, are required in order for AM80 to activate the invertebrate RAR. Indeed, the predicted *Lym*RAR ligand binding domain shares 53% amino acid identity with the ligand binding domain of *Homo sapiens* RARα.

These data, indicating that a synthetic RAR agonist (EC23) can mimic the effects of retinoic acid, are also supported by embryonic development studies in this molluscan species. *Lym*RAR protein is found in the developing embryonic CNS of *Lymnaea*, where it is found in axonal processes (Carter et al., 2015) as well as in the developing shell.

Interestingly, treatment of *Lymnaea* embryos with EC23 has been shown to exert similar effects on embryonic development as retinoic acid (mainly disrupting eye and shell formation) where AM80 had very few, to no effects (Johnson et al., 2019). Furthermore, RAR pan-antagonists (and selective RARβ antagonists) can disrupt *Lymnaea* embryonic development, again producing similar eye and shell defects (Carter et al., 2015). It should be noted that we have also previously shown that a vertebrate RAR pan-antagonist was capable of blocking growth cone turning induced by atRA in *Lymnaea* (Carter et al., 2015). Together, these pharmacological studies thus support a role for *Lym*RAR, though future studies will be required to confirm binding of *Lym*RAR to various vertebrate RAR agonists or antagonists.

Previous studies from our laboratory have shown that the ability of retinoic acid to induce growth cone turning in *Lymnaea* neurons, occurs in the absence of the cell body and thus in the absence of gene transcription (Carter et al., 2010; Farrar et al., 2009). These data suggest that, at least in this context, the effects of retinoic acid are non-genomic in nature. Indeed, retinoids are known to exert many non-genomic effects, including control of homeostatic plasticity in hippocampal cells (Aoto et al., 2008), as well as inducing rapid changes in intracellular calcium in *Lymnaea* neurons (Vesprini et al., 2015). Here, I provided immunostaining evidence for the localization of *Lym*RAR to neurites and growth cones of regenerating adult *Lymnaea* motorneurons, as well as a non-nuclear localization in the cultured neuronal cell bodies.

Previous Western blot analysis has also shown that RARs may be located in the membrane and cytoplasmic compartments of *Lymnaea* neurons (Carter et al., 2015).

Evidence from other studies suggest that receptors for guidance cues may be accumulated to the side of the growth cone closest to the chemoattractant during attractive growth cone turning (Bouzigues et al., 2007). Agonist data presented in this study suggest that retinoic acid might bind to RARs to initiate growth cone turning, although it remains to be determined whether RARs are redistributed in the growth cone (cytoplasm or membrane) during these turning responses. If these RARs are asymmetrically redistributed, future experiments can examine whether Rho GTPases might be involved in facilitating their transport (through cytoskeletal-dependent vesicle transport). Indeed, there is evidence that Rho GTPases transduce intracellular signals involved in vesicle trafficking and are involved in the regulation of receptor-mediated endocytosis (Chi et al., 2013; Lamaze et al., 1996).

In summary, using pharmacological analysis with vertebrate RAR agonists, I have shown a potential role of *Lym*RAR in mediating axon guidance.

4.2 The role of Rac in retinoic acid-induced growth cone guidance

Rho GTPases have emerged as important regulators of the actin cytoskeleton and increasing evidence has shown their involvement in growth cone responses to several guidance cues (Dickson, 2001). Many factors that influence axon guidance including slits, semaphorins, netrins, and ephrins have been shown to regulate Rho GTPase activity (Wong et al., 2001; Whiteford and Ghosh, 2001; Li et al., 2002; Wahl et al., 2000; Yuan et al., 2003). For example, Rac and Cdc42 (but not Rho) activity is required for netrin-induced neurite outgrowth (Li et al., 2002). Rho however, is required for the repulsive molecule ephrin to induce growth cone collapse (Wahl et al., 2000).

Here, I used cultured *Lymnaea* motorneurons and well-established growth cone turning assays to examine the role of Rho GTPases in retinoic acid-induced growth cone guidance. Previous research from our laboratory has shown that the intracellular pathway downstream of atRA is likely to involve Rac (Nasser, 2017). Specifically, growth cone turning towards atRA was abolished when Rac was inhibited by NSC23766. Furthermore, in the presence of NSC23766, atRA induced growth cone collapse and neurite retraction (Nasser, 2017). Here, I now show that the inhibition of Rac also abolished growth cone turning towards 9-*cis* RA. In this instance however, there was no growth cone retraction. Nevertheless, growth cone turning towards both isomers was abolished in the presence of the Rac inhibitor suggesting that the isomers are likely acting through, similar (or parallel) signalling pathways involving Rac.

Previously, it has been shown that growth cone guidance by retinoids can occur in the absence of the cell body, and thus in the absence of gene transcription, a response that is dependent on local protein synthesis (Farrar et al., 2009). Indeed, this was further confirmed in this study using both the all-*trans* and 9-*cis* retinoic acid isomers. However, one interesting finding from this study was that the involvement of Rac differed depending on whether the growth cones maintained communication with the cell body. I found that though the turning response of intact growth cones was blocked by Rac inhibition, isolated growth cones continued to respond to 9-*cis* RA in the presence of the Rac inhibitor. Similar evidence was provided by Nasser (2017), using the atRA isomer. That is, unlike intact growth cones, isolated growth cones continued to turn and advance towards atRA in the presence of the Rac inhibitor.

It is not yet known why the growth cone responses in the presence of the Rac inhibitor differed between intact and isolated growth cones. However, it is plausible that neurite transection could have impeded the effects of the Rac inhibitor. The Rac inhibitor may require transcriptional events to exert its effects, though this is unlikely, as it's normal mode of action is to interfere with GEF binding to Rac. It is perhaps more likely that the effects of the Rac inhibitor are dependent on communication with the cell body through the transport of regulatory proteins and signalling ligands. It is known that communication between the growth cone and cell body can be facilitated by propagating calcium waves from the growth cone to the cell body (Guan et al., 2007). Additionally, evidence has been provided that another path of communication involves the transport of vesicles formed in the growth cone to the cell body (Denburg et al., 2004). Although the exact mechanisms by which isolated growth cones continued to retain their responsiveness to retinoic acid in the Rac inhibitor is unclear, it may be that the inhibition of Rac facilitated by the Rac inhibitor requires growth cone communication with the cell body.

Another plausible explanation is that neurite transection initiated a compensatory mechanism to activate Rac. Guanine nucleotide exchange factors (GEFs) promote the conversion of GDP to GTP to enhance Rac activation (Dickson, 2001). The Rac inhibitor used in this study, NSC23766, inhibits Rac by targeting the Rac-specific GEFs, Trio and Tiam1 (Gao et al., 2004). It is therefore reasonable to speculate that growth cones isolated from the cell body may have relied on a compensatory mechanism involving alternative GEFs, which allowed the activity of Rac to persist. It is also possible that neurite transection initiated a compensatory mechanism that upregulated another protein involved in attractive growth cone guidance. It is known that there is a large influx of calcium

following neurite transection (Sattler et al., 1996), which can often induce growth cone collapse. This may trigger injury responses in the neurite and/or increases in calcium levels which might, in turn, activate other pathways. Either of these could result in upregulation of a protein that either facilitated growth cone turning towards retinoic acid or compensated for the inhibition of Rac (through a parallel or redundant mechanism).

Based on experimental data involving the inhibition of Rac, I conclude that Rac is a downstream effector of retinoic acid that is necessary to regulate positive growth cone turning towards retinoic acid. Rac is thought to mediate neurite extension and growth cone chemoattraction in response to other guidance cues (Dickson et al., 2001; Hall and Lalli, 2010). Consistent with this notion, I found that inhibition of Rac abolished growth cone turning towards a normally attractive gradient of retinoic acid (when growth cones are intact), although, the involvement of Rac in growth cone turning appeared to be dependent on the ability of the growth cone to communicate with the cell body. Nevertheless, these findings strengthen the previous conclusions that Rac is required for an axon to reach its appropriate target.

4.3 The role of Cdc42 in retinoic acid-induced growth cone guidance

Similar to Rac, the Rho GTPase Cdc42 is known for its role in growth cone chemoattraction and is thought to be important for axonal pathfinding and target recognition (Dickson et al., 2001; Hall and Lalli, 2010). For example, perturbing Cdc42 activity in cultured *Xenopus* spinal neurons abolishes chemoattraction induced by BDNF (Yuan et al., 2003) and ryanodine (which directly triggered intracellular calcium elevation) (Jin et al., 2005). However, its role in retinoic acid-mediated growth cone attraction has not

previously been studied. Here, I show that inhibiting Cdc42 blocked growth cone turning towards a gradient of atRA, and that this occurred in both intact and isolated growth cones. These data thus support previous findings that have also shown a role for Cdc42 in mediating chemoattractive growth cone guidance.

Importantly, the inhibition of Cdc42 in this study not only blocked retinoic acid-induced growth cone attraction, but also induced a switch in responsiveness from chemoattraction to chemorepulsion. A switch in responsiveness may allow growth cones to respond differently to the same guidance molecule at different stages of development or regeneration. I further confirmed that this switch in responsiveness was due to atRA and not merely an artifact of chemical application (by showing that growth cones did not exhibit repulsive turning to the vehicle in the presence of the Cdc42 inhibitor). Indeed, numerous studies indicate that whether a cue is attractive or repulsive can be modulated by various factors. For example, studies of cultured developing *Xenopus* neurons have demonstrated that inhibition of cyclic adenosine monophosphate (cAMP) causes an attractive growth cone response to netrin to be switched to repulsion (Ming et al., 1997; Nishiyama et al., 2003). Additionally, other studies have shown that inhibiting cAMP-dependent kinases (such as protein kinase A), in *Xenopus* growth cones can also switch BDNF-induced attraction to repulsion (Song et al., 1997; Yuan et al., 2003). Given the parallel effects on turning responses seen by inhibition of cAMP and Cdc42, it is possible that cAMP is acting upstream of Rho GTPases to facilitate the switch in responsiveness to atRA. It is thought that cAMP facilitates bi-directional growth cone responses by the modulation of calcium channel activity, which in turn, allows for differential activation of calcium-dependent effector proteins (Nishiyama et al., 2003; Sutherland et al., 2014).

It is intriguing to note that the Rac inhibitor also induced a switch in responsiveness, but only to the atRA isomer (Nasser, 2017) and not to 9-*cis* RA (current study). Thus, with both inhibition of Rac and Cdc42, atRA induced growth cone collapse and/or turning away, yet only blocked positive turning and did not induce significant repulsive responses to the 9-*cis* retinoic acid isomer. AtRA and 9-*cis* RA are biologically active isomers of retinoic acid and have both been identified in the CNS of *Lymnaea* at relatively similar concentrations (Dmetrichuk et al., 2008). Evidence has been provided that both isomers exert similar chemotropic effects to induce positive growth cone turning of *Lymnaea* motorneurons. However, differential effects of these retinoid isomers have also been shown in other studies. For instance, retinoid isomers have differing effects on the electrical properties of *Lymnaea* motorneurons, where atRA is capable of changing the firing properties of a cell, while 9-*cis* RA is not (Vesprini and Spencer, 2014). Additionally, atRA, but not 9-*cis* RA, is able to reduce the intracellular calcium levels and the intracellular calcium current through voltage-gated calcium channels of *Lymnaea* neurons (Vesprini et al., 2015, de Hoog et al., 2018). As the concentration of calcium is an important determinant of growth cone responses to many guidance cues, it is possible that the switch in growth cones responsiveness to atRA (but not 9-*cis* RA) observed here, is a result of reduced intracellular calcium levels in the growth cone induced by atRA (but not by 9-*cis* RA). Indeed, Rho GTPases function downstream of calcium to mediate growth cone turning, but interestingly, can themselves also influence calcium dynamics (Jin et al., 2005).

It is likely that numerous calcium-dependent effector proteins act upstream of Rho GTPases in the growth cone, which may possibly account for the different responses of

atRA and 9-*cis* RA when either Rac or Cdc42 are inhibited. It has been shown in other studies that localized calcium in the growth cone activates the calcium-sensitive kinases, calcium-calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC). Interestingly, inhibition of CaMKII has been shown to induce a switch in growth cone responsiveness to netrin (Wen et al., 2004), which parallels the switching of attraction to repulsion by the inhibition of Cdc42 found in this study and by the inhibition of Rac in Nasser (2017). Wen et al. (2004) demonstrated that localized calcium mediated different growth cone responses (attraction versus repulsion) by differential activation of CaMKII and calcineurin, respectively. Thus, atRA decreasing intracellular calcium may result in less activation of CaMKII and increased activation of calcineurin, compared to 9-*cis* RA. This differential activation of calcium-dependent effector proteins may account for the switch in growth cone responsiveness to atRA, but not to 9-*cis* RA. Furthermore, Jin et al. (2005) have demonstrated that the signal transduction from calcium to Cdc42 in netrin-induced growth cone guidance is mediated by PKC and requires basal activity of CaMKII. It is therefore conceivable that CaMKII and PKC may act upstream of Cdc42 to control the direction of growth cone turning induced by retinoic acid and that retinoid isomers result in differential activation of calcium-dependent proteins due to their differential abilities to affect calcium influx and/or intracellular calcium levels. Clearly, future studies are required to identify the role of CaMKII and PKC in growth cone turning induced by both retinoid isomers.

In this study, both isomers were tested in the Rac inhibitor, but so far only atRA has been examined in the Cdc42 inhibitor. In order to determine whether the switch in responsiveness is an isomer-dependent effect, experiments with 9-*cis* RA in the presence

of the Cdc42 will need to be completed. Indeed, preliminary data from our laboratory now suggest that 9-*cis* RA does not induce a switch in growth cone responsiveness. Additionally, the question of what downstream targets(s) of Rac and Cdc42 are involved in retinoic acid-mediated growth cone guidance also remains to be addressed. Potential effectors of Rac and Cdc42 include WASP proteins, which promote actin formation (Govek et al., 2005), and p21-acitvated kinases, whose substrates include cytoskeletal regulators (Bokoch, 2003).

Previous studies have demonstrated that genetic disruption of Rho GTPases leads to axon pathfinding defects (Hall and Lalli, 2010). Expression of either constitutively active (constantly active) or dominant-negative forms of Rho GTPases (mutation that blocks protein function), resulted in axons guidance errors in *Xenopus*, *Drosophila,* and *C. elegans* (Ruchhoeft et al., 1999; Ng et al., 2002; Lundquist et al., 2003), suggesting that precise regulation of Rho GTPases is crucial for proper growth cone guidance. My findings provide further evidence for the role Rho GTPases in growth cone guidance, as I show here that the intracellular pathways downstream of retinoic acid likely involve Rac and Cdc42. These studies also provide further evidence that retinoic acid requires many of the same signalling pathways as traditional guidance cues, such as semaphorins and netrins.

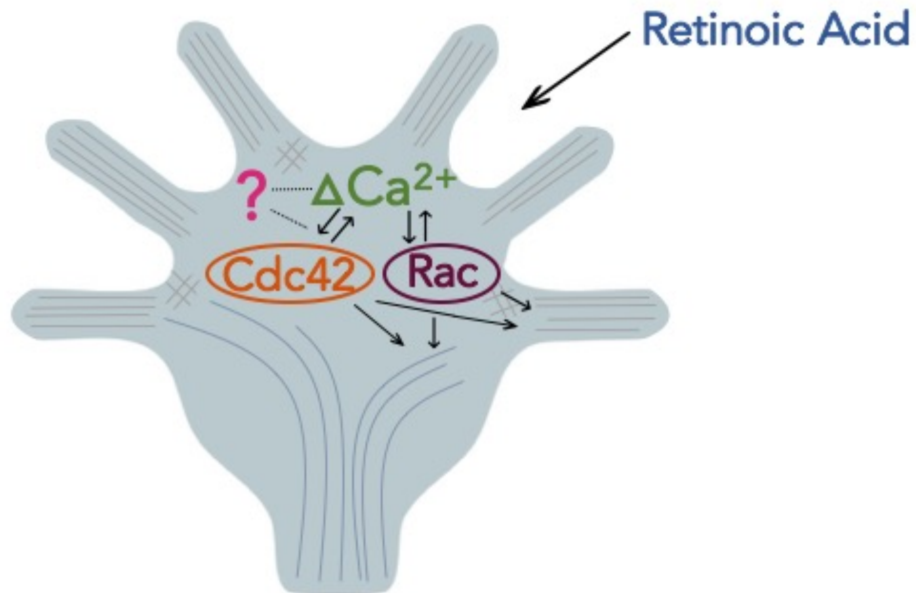
4.4 Conclusions

The process of growth cone guidance is one that is multifaceted, involving extracellular guidance cues, intracellular signalling cascades, and cytoskeletal rearrangements. While progress has been made in the identification of guidance molecules and their receptors, gaps still remain in the understanding of intracellular signalling

pathways that regulate growth cone dynamics. Here, I have identified a role for the Rho GTPases, Rac and Cdc42, as important signalling molecules for mediating the chemotropic effects of retinoic acid. These findings reveal a mechanism in which Rac and Cdc42 are downstream effectors of retinoic acid that regulate growth cone guidance. Importantly, I have shown that the involvement of Rac differed depending on whether the growth cones have maintained communication with the cell body or not. Moreover, the inhibition of Cdc42 not only blocked growth cone turning towards retinoic acid, but also induced a switch in growth cone responsiveness from attraction to repulsion. Together, my findings indicate a model (Fig. 4.1) in which Rho GTPases control the directional motility of growth cones in response to retinoic acid.

Although much is known about the ability of retinoids to mediate neurite outgrowth during nervous system development, the cellular and molecular mechanisms underlying growth cone pathfinding by retinoids remain largely unknown. Understanding the signalling cascades and downstream effectors of retinoic acid is a crucial step to understanding axon pathfinding. In collaboration with the Carlone laboratory at Brock University, we are currently the only research group (to our knowledge) investigating the signal transduction pathways underlying retinoic acid-induced growth cone guidance. Insights derived from these studies will enhance our basic understanding of neuronal pathfinding during both development and regeneration.

A



B

atRA	*Rac Inhibitor (NSC23766)*	*Cdc42 Inhibitor (ML141)*
intact	–	–
isolated	+	X
9-cis RA		
intact	X	TBD
isolated	+	TBD

+ Attraction
– Repulsion
X Response Blocked

Figure 4.1: Schematic diagram illustrating the proposed role of Rho GTPases in retinoic acid-induced growth cone guidance. (**A**) A gradient of retinoic acid requires calcium influx within the growth cone. Calcium can act on unknown effectors (although potential candidates include CaMKII and PKC), which then act on Rho GTPases to regulate the dynamics of actin and microtubules. Alternatively, calcium may act directly on Rho GTPases and in turn, Rho GTPases may also influence the calcium signal. Retinoid receptors (not shown) are localized within the growth cone, likely in the membrane and cytoplasm. (**B**) Summary table of the experimental growth cone responses to atRA and 9-*cis* RA in the presence of the Rac or Cdc42 inhibitor. (AtRA data in the presence of the Rac inhibitor taken from Nasser, 2017).

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