Retinoic acid and the Underlying Cellular Mechanisms Involved in Neurite Outgrowth and Growth Cone Turning during Regeneration

Tamara I. N. Nasser, Hon. B.Sc.

A thesis submitted to the Department of Biological Sciences in partial fulfillment of the requirements for the degree of Master of Science

Faculty of Mathematics and Science,
Brock University
St. Catharines, Ontario

© Tamara I.N. Nasser, 2017
Abstract

The involvement of retinoic acid (RA) in nervous system regeneration has been well documented, though the precise cellular and molecular mechanisms have not yet been fully determined. During regeneration, RA can exert trophic support for cells, as well as tropic effects to guide neurite outgrowth. Cultured neurons of the Lymnaea central nervous system (CNS) have previously been used to investigate the role of RA in neurite outgrowth and growth cone guidance. Recently however, a novel phenomenon has been identified, in which neurite outgrowth occurs from cut nerves of Lymnaea CNS “floating” on the surface of cultured medium. In this study, I examined whether RA could induce or guide neurite outgrowth from this novel preparation. Unlike previous findings with cultured neurons, there was a lack of consistent effects of 9-cis and all-trans RA in promoting neurite outgrowth from the floating CNS. However, the growth cones of these floating neurites were found to turn toward a local source of RA, indicating for the first time, that they are capable of responding to guidance cues, even in the absence of adhesion to a solid substrate. The cellular mechanisms of RA-induced growth cone turning were then conducted on cultured neurons. I demonstrated that various retinoid receptor agonists could mimic the chemotropic effects of RA, providing further evidence for a potential role of retinoid X receptor and retinoic acid receptors in growth cone turning. I also examined potential downstream effectors involved in this chemotropic response to RA and provided the first evidence that the intracellular signaling pathway likely involves the Rho GTPase, Rac. However, it was also shown that the involvement of Rac in mediating the RA-induced growth cone turning differed depending on whether the turning was induced by endogenous or synthetic retinoids, and also whether the growth cones were still attached to the cell body or not. Overall, these data provide new insights into the mechanisms by which retinoids affect neurite outgrowth and growth cone behaviour during regeneration of the nervous system.
Acknowledgements

I would first like to thank my supervisor, Dr. Gaynor Spencer, for the support and guidance she has provided me throughout the course of my Master’s degree. It has been an honor working in her lab. I would like to thank her for this opportunity, and for her patience and commitment to not only my research but also throughout the writing process of this thesis.

I would like to thank the members of my thesis committee, Dr. Glenn Tattersall and Dr. Robert Carlone, for their helpful suggestions and insights during the course of my master's degree program. I would also like to thank Dr. Tattersall for the use of the oxygen regulator and for kindly taking the time to provide statistical advice. In addition, I would like to thank Dr. Necakov for his helpful suggestions on analyzing data. I also appreciate the time and effort of the faculty who taught me in my graduate courses (Drs. Spencer, Carlone, Mercier, McCormick, Liang, and Despres). Additionally, I would like to formally thank Cailin Rothwell and Nick Vesprini for their time and effort assisting me throughout my graduate studies.

Without question, the most influential people in my life have been my family. I would like to thank them very much for their unconditional love and support. Thank you for all that you have done, and all that you continue to do for me. I am forever grateful and blessed to have you as my family.

Finally, I would like to thank my laboratory colleagues including, Sarah Walker and Eric de Hoog, who have been a source of ongoing support and assistance.

Thank you.
Table of Contents:

Abstract II
Acknowledgements III
Table of Contents IV
List of Figures VII
List of Abbreviations IX

1. Introduction and Literature Review 1
   1.01 General Introduction 2
   1.02 Retinoic Acid Synthesis 3
   1.03 Retinoic Acid Signalling 4
   1.04 The Role of Retinoid Signalling During Development 6
   1.05 The Role of Retinoid Signalling During Regeneration 7
   1.06 The Role of Retinoic Acid in Axon Guidance 9
   1.07 Local Response of Growth Cones 11
   1.08 Rho GTPases, Potential Downstream Effectors in Growth Cone Signalling 12
   1.09 Retinoid Signalling in *Lymnaea stagnalis* 16
   1.10 Retinoid Receptors are conserved between vertebrates and invertebrates 17
   1.11 *Lymnaea stagnalis* as a Model to Study of the Role of Retinoic Acid in Regeneration 19
   1.12 A Novel Preparation: The Floating *Lymnaea CNS* 20
   1.13 Thesis Objectives 22

2. The Effect of Retinoids on Neuronal Outgrowth from the Novel Floating Central Nervous System Preparation 23
   2.01 Introduction 24
   2.02 Materials and Methods 26
Animals 26
Chemicals 26
Floating CNS Preparation 26
Growth Cone Turning Assays 27
Data and Statistical Analysis 28

2.03 Results 32
The Effect of Retinoids on Neurite Outgrowth 32
atRA Induced Growth Cone Turning of Floating Neurites 39

2.04 Discussion 41
The Effect of Retinoids on Neuronal Outgrowth from the Novel Central Nervous System Preparation 41
The Effect of atRA on the Growth Cone Turning Behaviour of Floating Neurites 44

3. The Effect of Retinoids on Neuronal Outgrowth from the Novel Floating Central Nervous System Preparation 47

3.01 Introduction 48
3.02 Materials and Methods 51
Animals 51
Preparation of Poly-l-Lysine Coated Culture Dishes and Lymnaea Conditioned Medium (CM) 51
Cell Culture Procedures 52
Chemicals 54
Growth Cone Turning Assays 55
Growth Cone Measurements 57
Data and Statistical Analysis 57

3.03 Results 58
The effects of synthetic retinoids on growth cone turning in the pond snail, *Lymnaea stagnalis*

Growth cones turn towards a gradient of atRA

Growth cones turn towards a gradient of EC23

CH55 induces variable effects on growth cone behaviour

Growth cones turn towards a gradient of SR11237, a selective RXR pan agonist

The Role of Rho GTPase Rac in atRA-induced Growth Cone Turning

The Role of Rac in EC23-induced Growth Cone Turning

The Rac Inhibitor Does Not Inhibit atRA-Induced Growth Cone Turning of Isolated Growth Cones

3.04 Discussion

The potential role of *LymRAR* and *LymRXR* in retinoic acid-induced growth cone turning

The role of Rac in atRA-induced growth cone turning

The role of Rac in EC23-induced growth cone turning

The role of Rac in atRA-induced turning of isolated growth cones

4. Conclusions and Perspectives

Appendix

References
List of Figures

Figure 1. The retinoic acid synthesis pathway 4
Figure 2. Retinoic acid’s classical mechanism of action in the cell nucleus. 5
Figure 3. The neuronal growth cone. 10
Figure 4. Rho GTPases regulate cytoskeleton remodelling 15
Figure 5. Neurite outgrowth from a floating *Lymnaea* nerve. 21
Figure 6. *Lymnaea* CNS. 27
Figure 7. Analysis of the proportion of neurite outgrowth coverage within a defined area. 30
Figure 8. Measurement of the turning angle of floating growth cones in response to atRA. 31
Figure 9. Neuronal outgrowth from floating CNS in DMSO, 9-cis RA, and atRA. 33
Figure 10. Fewer nerves exhibit neurite outgrowth in atRA at lower concentrations. 34
Figure 11. Retinoids do not increase the mean length of neurite outgrowth. 36
Figure 12. 9-cis RA induces more neurite coverage than atRA. 38
Figure 13. Floating growth cones are attracted to a gradient of atRA. 40
Figure 14. *Lymnaea* CNS prepared for neuron extraction 53
Figure 15. Schematic of the cell isolation process and assembly. 54
Figure 16. Representative example of Pedal A neuronal outgrowth in culture. 55
Figure 17. A neurite mechanically isolated from its cell body. 56
Figure 18. Growth cones are attracted to a gradient of atRA but fail to turn towards the vehicle. 59
Figure 19. Growth cones are attracted to a gradient of the synthetic retinoid, EC23. 61
Figure 20. CH55 induces variable effects on growth cone behaviour. 62
Figure 21. Growth cones are attracted to a gradient of the SR11237. 64
Figure 22. Summary of growth cone responses to retinoids. 66
Figure 23. A switch in the growth cone turning response to atRA in the presence of the Rac inhibitor. 69
Figure 24. Growth cones fail to turn towards atRA in the presence of Rac inhibitor. 70
**Figure 25.** Rac inhibitor blocks growth cone advancement in response to atRA application

**Figure 26.** Rac inhibitor does not block EC23 mediated growth cone turning.

**Figure 27.** Rac inhibitor blocks growth cone turning in response to atRA but not in response to EC23.

**Figure 28.** Rac inhibitor blocks growth cone advancement in response to atRA but not in response to EC23.

**Figure 29.** Rac inhibitor does not block isolated growth cone turning towards atRA.

**Figure 30.** Rac inhibitor does not block transected growth cone turning towards atRA.

**Figure 31.** Rac inhibitor blocks growth cone advancement of intact, but not transected, neurites in response to atRA.

---

**Appendix:**

**Appendix 1.** Fewer nerves exhibit neurite outgrowth at lower concentrations of atRA.

**Appendix 2.** Retinoids do not increase the mean length of neurite outgrowth.

**Appendix 3.** Description of the retinoid receptor agonists used in this study.

**Appendix 4.** Transactivation Activity of RAR/RXR selective retinoids.

**Appendix 5.** Methods for measuring the proportional area occupied by regenerative processes (%) in ImageJ software.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABS</td>
<td>Antibiotic saline</td>
</tr>
<tr>
<td>ADH</td>
<td>Alcohol dehydrogenase</td>
</tr>
<tr>
<td>ARP 2/3</td>
<td>Actin-related protein 2/3</td>
</tr>
<tr>
<td>atRA</td>
<td>All-trans retinoic acid</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cell division control protein 42</td>
</tr>
<tr>
<td>9-cis RA</td>
<td>9-cis retinoic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRABP</td>
<td>Cellular retinoic acid-binding proteins</td>
</tr>
<tr>
<td>CRBP</td>
<td>Cellular retinol-binding protein</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CYP26</td>
<td>Cytochrome P450 enzyme</td>
</tr>
<tr>
<td>DM</td>
<td>Defined Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating proteins</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>mDia</td>
<td>Mammalian diaphanous</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>PeA</td>
<td>Pedal A</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>Rac</td>
<td>Ras-related C3</td>
</tr>
<tr>
<td>RALDH</td>
<td>Retinal dehydrogenase</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic Acid Receptor</td>
</tr>
<tr>
<td>RARE</td>
<td>Retinoic acid response element</td>
</tr>
<tr>
<td>RBP</td>
<td>Retinol-binding protein</td>
</tr>
<tr>
<td>Rho</td>
<td>Ras homolog</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho Kinase</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
</tr>
<tr>
<td>STRA6</td>
<td>Stimulated by retinoic acid gene 6</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASP family verprolin homologous protein</td>
</tr>
</tbody>
</table>
Chapter 1:

Introduction and Literature Review
1.01 General Introduction

The establishment of precise connections in the central (CNS) and peripheral (PNS) nervous system throughout regeneration is important for their correct functioning. During regeneration, damaged nervous tissues regrow and repair. Retinoic acid is a molecule that has been implicated in exerting trophic effects to support the outgrowth of neurites during regeneration (Dmetrichuk et al. 2005; Dmetrichuk et al. 2006). A trophic effect refers to the stimulation of growth and/or elongation of neurites. Additionally, retinoic acid can exert tropic effects and act as a chemoattractant in vertebrates and invertebrates (Dmetrichuk et al., 2005; 2006). A chemoattractant is a chemical molecule that can guide neurite processes (usually toward an appropriate target) during development or regeneration, thus generating a tropic (or turning) response. The involvement of retinoic acid in the regeneration of both the CNS and PNS has been well documented (Maden 2007), though its precise role is yet to be determined. Hence, it is essential to gain a better understanding of the underlying mechanisms by which retinoic acid directs neurite outgrowth during regeneration.

In contrast to the adult mammalian CNS, adult invertebrates such as the mollusc Lymnaea stagnalis retain the ability to regenerate CNS neurons. Also, Lymnaea possesses a relatively simplistic and well-characterized nervous system and is a well-established model system to study regeneration. We have used Lymnaea to study both the trophic and chemotropic effects of retinoic acid on neurite outgrowth from the CNS and from cultured neurons. Comparing the effects of retinoid signalling in the regenerating invertebrate nervous system with its various effects in vertebrates, may allow us to gain a better understanding of its role in enhancing and guiding neurite outgrowth.
Literature Review:

1.02 Retinoic Acid Synthesis

Retinoic acid is the most biologically active, naturally occurring member of a family of retinoids derived from Vitamin A. Retinoic acid is obtained from the diet in the form of carotenoids (from the intake of fruits and vegetables), or in the form of retinyl esters (from the tissue of animals that have already ingested and metabolised carotenoids). These compounds are then stored in the body, mainly in the liver, in the form of retinyl esters (Blomhoff and Blomhoff, 2006). When required by cells, the stored retinyl esters are converted to retinol and bind to retinol-binding proteins (RBPs) for transport in the bloodstream to specific target areas (Maden, 2007). Retinol then enters the cell, via a transmembrane protein known as STRA6 (stimulated by retinoic acid gene 6), and binds to cellular RBPs (CRBPs) (Niederreither and Dollé, 2008). CRBPs facilitate the conversion of retinol to retinal via alcohol dehydrogenase (ADH). The enzyme retinal dehydrogenase (RALDH) then irreversibly metabolizes retinal to retinoic acid via oxidation (Duester, 2000). Cellular retinoic acid-binding proteins (CRABPs) can facilitate the transport of retinoic acid within the cell (autocrine signaling), or out of the cell (to signal in paracrine manner). In the autocrine signaling process, retinoic acid can enter the nucleus and bind to ligand activated nuclear transcription factors, known as retinoid receptors. These retinoid receptors bind to a sequence of DNA called a retinoic acid-response element (RARE), and with the aid of a range of co-activators or co-repressors, can ultimately lead to changes in gene activity. The important function of controlling the levels of retinoic acid in the body is established by enzymes from the CYP26 family (cytochrome P450 enzymes), which can catabolise retinoic acid into polar metabolites (Reijntjes et. al., 2005; Figure 1).
Figure 1. The retinoic acid synthesis pathway. Vitamin A (retinol) can be stored in the form of retinyl esters or transported into cells and converted via a metabolic reaction, involving alcohol dehydrogenases (ADHs) and retinaldehyde dehydrogenases (RALDHs), into retinoic acid. Retinoic acid can then act in an autocrine fashion and regulate gene transcription in the cell that synthesized it, or it can act in a paracrine manner, regulating the transcriptional activity of genes in neighboring target cells. The degradation of retinoic acid by Cyp26 enzymes serves to control the levels of retinoic acid in tissues (Modified from Niederreither and Dolle, 2008).

1.03 Retinoic Acid Signalling

Retinoic acid’s primary mode of action thus involves signalling through the nuclear receptors known as retinoic acid receptors (RARs) and retinoid X receptors (RXRs). RARs and RXRs are thought to be activated by the binding of different retinoic acid isomers (retinoids with the same molecular formula but different chemical structures). Studies on vertebrates have found that RARs can be activated by both 9-cis and all-trans retinoic acid (Allenby et. al., 1994). RXRs almost exclusively bind 9-cis retinoic acid, though all-trans retinoic may bind to some RXRs but with lower affinity compared to its binding to RARs (Heyman et. al., 1992). Once bound to the receptors, the ligands can induce
heterodimerization of RAR and RXR. RXR can also function as a homodimer, or RXR can also form heterodimers with other receptors such as the Vitamin D and thyroid receptors (Yu et al., 1991). Hence, the possibility for homodimerized activity of RARs and RXRs exists, in which each receptor is individually activated by its respective ligands (Figure 2).

Figure 2. Retinoic acid’s classical mechanism of action in the cell nucleus. This involves binding to nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Vertebrate RXRs demonstrate a preference for 9-cis retinoic acid, whereas RARs can be activated by both 9-cis and all-trans retinoic acid. All-trans retinoic acid may also bind to RXRs, but at a lower affinity (represented by the dotted arrow) compared to RARs. Following the binding of retinoic acid, these receptors heterodimerize or homodimerize then bind to a retinoic acid-response element (RARE) sequence to regulate gene activity (Modified from Simões-Costa et al., 2008).

The two active isomers of retinoic acid, all-trans and 9-cis retinoic acid, have been found to exert different effects in some cases, whereas others have determined that both isomers can act similarly. Pedersen et al. (1995) showed that both isomers increased the choline acetyltransferase activity and the intracellular levels of acetylcholine in a murine septal cell line. Furthermore, all-trans and 9-cis retinoic acid induced similar inhibition of proliferation of human neuroblastoma cells (Plum and Clagett-Dame, 1995) and induced
similar neurotrophic effects on outgrowth from cultured molluscan neurons (Dmetrichuk *et. al.*, 2008).

In contrast, other studies have determined that all-*trans* and 9-*cis* retinoic acid can act in a different manner, either in the concentration required or in their effects. For example, Han *et. al.* (1995) found 9-*cis* retinoic acid to be more potent than all-*trans* retinoic acid in its antiproliferative and differentiation activity in human neuroblastoma cells, and both isomers were found to exert different effects on the production of extracellular matrix proteins and the proliferation of activated hepatic stellate cells from rats (Hellemans *et. al.*, 1999). All-*trans* retinoic acid exerted inhibitory effects on the synthesis of extracellular matrix proteins and did not influence the proliferation of these stellate cells. In contrast, 9-*cis* retinoic acid upregulated procollagen I mRNA but did not affect the synthesis of other extracellular matrix proteins, and inhibited the proliferation of the hepatic stellate cells (Hellemans *et. al.*, 1999). Furthermore, all-*trans* retinoic acid was found to be more potent in inducing abnormal firing activity and reducing intracellular calcium levels of cultured molluscan neurons (Vesprini and Spencer, 2014; Vesprini *et. al.*, 2015).

1.04 The Role of Retinoid Signalling During Development

Retinoic acid has been established to play an essential role in neuronal patterning, neural differentiation and axon outgrowth, and contributes to the anteroposterior and dorsoventral patterning of the neural plate and neural tube (Maden, 2007). Retinoic acid has been detected at various developmental stages in several species, including mouse and chick embryos (Chen and Solursh, 1992; Maden, 2000) and axolotl embryos (Monaghan and Maden, 2012), and its levels increase during the development of *Xenopus* embryos (Chen *et. al.*, 1994). Several experimental approaches have not only identified and emphasized the
presence of retinoic acid during development, but also its regulation. Excess retinoic acid has been shown to cause teratogenesis, targeting several areas of the body including the hearts of avian embryos, rats, and hamsters, and the limbs of developing chick, mice, and urodeles (Ross et. al., 2000; Zile, 2001). Alternatively, nutritional deficiencies in vitamin A as well as retinoid receptor null mutants have led to severe abnormalities in the face, neural crest, heart, and nervous system of rats (Dickman et. al., 1997). Similarly, abnormalities in the formation of the neural crest and hindbrain, the visual and auditory systems, and several other organs were observed in quail, mice and rats, following knock-down of the retinoid receptors (Ross et. al., 2000; Zile, 2001).

1.05 The Role of Retinoid Signalling During Regeneration

Retinoic acid has not only been established to play an essential role in development, but also in tissue regeneration following injury or amputation. Some lower vertebrates (Prince and Carlone, 2003; Dmetrichuk et. al., 2005) and many adult invertebrates (Syed et. al., 1990; Dmetrichuk et. al., 2006), such as the mollusc Lymnaea stagnalis, have retained the ability to regenerate their CNS. However, adult neurons of the mammalian CNS, unlike the PNS, fail to regenerate. Factors that have been found to contribute to the inability of neurites to regenerate in the mature CNS include the presence of inhibitory components, such as Nogo and myelin-associated glycoprotein (Mag) (Baldwin and Giger, 2015), as well as a decrease in the level of neurotrophic factors present. Moreover, the downregulation of retinoic acid signalling is thought to be involved in the loss of regenerative capacity of some adult CNS neurons in vertebrates (Maden, 2003). For example, retinoic acid was found to induce neurite outgrowth in mouse embryonic neurons and spinal cord but failed to do so in adult spinal cord. Yet, when the retinoic acid receptor gene, RARβ2, was introduced into the
adult mouse spinal cord, neurite outgrowth was observed (Corcoran et al., 2002). Hence, these data have suggested an important role for retinoic acid signalling in regeneration.

Further evidence supporting the involvement of retinoic acid in regeneration includes the observation that retinoic acid synthesis and signalling are up-regulated following injury in several species. The local synthesis and release of both 9-cis retinoic acid and all-trans retinoic acid was found in the wound epidermis of the regenerating limbs of newts (Viviano et al. 1995). An increase in the activity of the enzyme retinal dehydrogenase 2 (RALDH-2) and an upregulation of the cellular retinol-binding proteins-1 and 2 (CRBP-1, CRBP-2) components of the retinoic acid signalling pathway, were observed following sciatic nerve crush injury in adult rats (Zhelyaznik et al., 2003). Moreover, experiments with axolotls (Maden, 1982; 1983) showed a concentration-dependent effect of retinoic acid on the amount of tissue regenerated in the proximodistal axis. Retinoic acid signalling was also detected during limb regeneration in the apical epidermis, nerves, and nerve-associated cells in transgenic axolotls (Monaghan and Maden, 2012).

Evidence from various model systems including newt spinal cord tissue (Dmetrichuk et al., 2005), embryonic chick dorsal root ganglion neurons (Maden, 1998) and quail embryonic spinal cords (Maden et al., 1996) have also implicated the role of retinoic acid in inducing and enhancing neurite outgrowth. Furthermore, in addition to the neurotrophic effects of retinoic acid, retinoic acid has also been determined to act as a tropic factor to guide neurite outgrowth from regenerating tissue.
1.06 The Role of Retinoic Acid in Axon Guidance

The guidance of neurites to their appropriate targets is accomplished by dynamic structures called growth cones, located at the tips of extending neurites. These structures are comprised of two regions, the central and peripheral region. The central region consists of microtubules and various organelles required for neurite extension (Lowery and Van Vactor, 2009; Dent et. al., 2011). The second region, the peripheral region, consists of the actin filaments required for membrane protrusion. These actin filaments undergo polymerization with the addition of actin monomers on one end of the actin filament and depolymerization on the other end. This helps to establish the protrusive forces required at the leading edge of the growth cone. Additionally, adhesion complexes, involving actin-binding proteins, mediate connections between actin and the plasma membrane, and the motor protein myosin II generates the traction forces required for growth cone advancement (Gomez and Letourneau, 2014).

The actin filaments in the peripheral region are organised into bundles in the filopodia but exist as a meshwork in the lamellipodia (Bamburg et. al., 1992). Filopodia appear as finger-like projections whereas lamellipodia appear as veil-like projections (Figure 3). These projections possess membrane receptors at their tips and consistently protrude and withdraw in order to sense the numerous guidance cues in their environment (Gomez and Letourneau, 2014). These guidance cues can be attractive or repulsive, inducing growth cone turning toward the cue and subsequent neurite extension, or causing growth cone collapse and subsequent neurite retraction and/or turning away from the cue, respectively.
Figure 3. The neuronal growth cone. The central region (C) of the growth cone is comprised of microtubules and various organelles. The polymerization of microtubules at the positive (+) end contributes to neurite outgrowth. The peripheral region (P) consists of actin filaments organised in bundles in the filopodia and as a meshwork in the lamellipodia. The actin filaments are required for membrane protrusion. Taken from Mueller (1999).

Numerous factors are known to act as guidance cues, and these include chemicals such as netrins, semaphorins, slits, ephrins, neurotrophins, cytokines, neurotransmitters, classical morphogens, growth factors, extracellular matrix molecules, as well as retinoic acid (Farrar and Spencer, 2008). Electrical fields can also affect growth cone behaviour and the direction of outgrowth (Rajnicek et al., 2006).

Maden et al. (1996) first suggested a chemotropic role for retinoic acid in guiding neurite outgrowth, after finding abnormal neurite trajectory in the developing neural tube of Vitamin-A deficient quail embryos. Evidence supporting this was provided in 1998 when a Dunn chamber was used to generate a stable gradient of all-trans retinoic acid for cultured chick dorsal spinal cord neurons. When the gradient was encountered, the regenerating neurites changed their orientation and extended towards the higher concentration of retinoic acid (Maden et al., 1998). More recently, when newt spinal cord explants were co-cultured...
with a newt limb blastema, many of the neurites extended in the direction of the blastema. Neurite outgrowth from the newt spinal cord explants, however, was significantly reduced in the presence of a retinoic acid synthesis inhibitor, citral, suggesting that retinoic acid was one of the molecules promoting the outgrowth of the neurites towards the blastema (Prince and Carlone, 2003). Similarly, Dmetrichuk et. al. (2005) demonstrated that beads soaked in retinoic acid and co-cultured with newt spinal cord explants induced preferential directed neurite outgrowth towards the source of beads. Altogether, these findings suggest a crucial role for retinoic acid in both development and regeneration, particularly in axon guidance.

1.07 Local Response of Growth Cones to Guidance Cues

Growth cones, mechanically isolated from their cell bodies, have been shown to retain their capacity to respond to chemical guidance cues. Preliminary evidence for a local response of growth cones was established when isolated growth cones of chick sensory ganglion cells were shown to survive and continue to grow in culture (Shaw and Bray, 1977). Further evidence was provided when retinal axons of embryonic Xenopus laevis continued to display normal pathfinding behaviour, in vivo, following the removal of their somata (Harris et. al., 1987), and continued to respond to chemical guidance cues, in vitro (Brunet et. al., 2005). However, chemotropic guidance of these growth cones was lost following inhibition of protein synthesis (Campbell and Holt, 2001). Altogether, these findings suggest that a growth cone can function as a semi-autonomous structure and may rely on local protein synthesis in order to respond to various guidance cues.

In fact, key components of the translational machinery (mRNA and ribosomes) are present in growth cones which have been shown to be capable of local protein synthesis (Campbell and Holt, 2001; Wu et. al., 2005). For example, the chemical guidance cues
netrin-1 and Sema3A activate translation initiation factors and stimulate a marked rise in protein synthesis in isolated retinal growth cones from *Xenopus laevis* (Campbell and Holt, 2001). Chemotropic guidance of these growth cones was lost following the inhibition of either protein synthesis or mRNA translation (Campbell and Holt, 2001). Additionally, several mRNA transcripts are found localized to growth cones of many different types of neurons, including rat dorsal root ganglion and hippocampal neurons (Zhang *et al.*, 1999; Willis *et al.*, 2005), spinal neurons (Yao *et al.*, 2006) and sympathetic neurons (Lee and Hollenbeck, 2003). Particularly relevant to the research conducted in this thesis, Farrar *et al.* (2009) showed that retinoic acid-mediated attractive growth cone turning does not require gene transcription (and thus involves non-genomic retinoid signalling). Using cultured *Lymnaea* motorneurons, it was shown that transected neurites (removed from the cell body) continued to respond to retinoic acid. Furthermore, it was shown that retinoic acid-induced chemoattraction, was dependent on *de novo* local protein synthesis. Many studies to date, have thus established a level of autonomy for growth cones in responding to guidance cues, and recent data have now shown the same is true for growth cones responding to retinoic acid.

**1.08 Rho GTPases, Potential Downstream Effectors in Growth Cone Signalling**

In addition to protein synthesis, the importance of calcium signaling in growth cone decisions has been well established (Henley and Poo, 2004). The direction of growth cone turning is often dependent on the characteristics of the calcium signals, such as amplitude, frequency, localization in the growth cone, and source of the calcium (Gomez and Zheng, 2006). Calcium changes in the growth cones can be generated by calcium release from intracellular stores or influx through plasma membrane channels (or both) (Gomez and
Zheng, 2006). Calcium-mediated second messenger signalling pathways in the growth cone have been shown to regulate the actin cytoskeleton, thereby modulating the type of behavior exhibited upon contact with certain guidance factors (Gomez and Letourneau, 2014).

The signaling cascades which link the growth cone calcium changes to the cytoskeletal movement require further research. One link that has been proposed is a family of small guanosine tri-phosphatase binding proteins called Rho GTPases, which includes Ras homolog (Rho), Ras-related C3 (Rac) and cell division control protein 42 (Cdc42). Rho GTPase signalling occurs downstream of activation of membrane receptors and calcium signals, and upstream of effectors of cytoskeletal dynamics. For example, Rho GTPases can affect the activity of proteins that regulate the assembly of the actin cytoskeleton, including actin-binding proteins (Hall, 2005). Rho GTPases can be activated by numerous guidance factors which include semaphorins (Whitford and Ghosh, 2001), netrins (Li et al., 2002), slits (Wong, et al., 2001), ephrins (Wahl et al., 2000), and brain-derived neurotrophic factor (BDNF) (Yuan et al., 2003). This involvement of Rho GTPases in growth cone responses has also been widely observed across many species such as Drosophila (Whitford and Ghosh, 2001), mice (Li et al., 2002), rats (Wong, et al., 2001), chicken (Wahl et al., 2000), and Xenopus laevis (Yuan et al., 2003). Specifically, genetic disruption of GTPase activity has resulted in growth cone pathfinding defects in Drosophila (Kaufman et al., 1998) and Xenopus (Rajnicek et al., 2006) embryos. However, whether Rho GTPase signal transduction pathways regulate cytoskeletal dynamics during retinoic acid-induced growth cone guidance has not yet been examined in any species.

Spatial and temporal regulation of Rho GTPases in the growth cones contribute to both positive and negative turning responses to guidance cues. Rho GTPases are activated when guanine nucleotide exchange factors (GEFs) catalyze the exchange of guanosine
diphosphate (GDP) for guanosine triphosphate (GTP). They are inactivated upon hydrolysis of GTP by GTPase activating proteins (GAPs) (Gao et al., 2004). Cdc42 and/or Rac are activated on the chemoattractant-facing side of the growth cone, whereas Rho is less active. Activation of Cdc42 and Rac stimulates the formation of filopodia and lamellipodia, respectively, thereby promoting microtubule elongation and dynamics on the chemoattractant side (Kozma et al., 1997). The mechanisms through which Rho GTPases likely regulate the growth cone assembly of the cytoskeleton are still not clear but several effectors have been suggested. Rac and Cdc42 have been shown to interact with WAVE (WASP family verprolin homologous protein) and WASP (Wiskott–Aldrich syndrome protein)-related proteins, respectively, which can activate the Arp2/3 (actin-related protein 2/3) complex to promote actin polymerization (Hall, 2005). Rac has also been found to act through cofilin (an actin-binding protein), to promote protrusion at the front of migrating cells (Gomez and Letourneau, 2014).

During chemorepulsion, the reverse is believed to occur, and Rho is more active. Rho has been found to act through Rho kinase (ROCK) to phosphorylate myosin light chain and inhibit the myosin light chain phosphatase. Inhibition of the myosin light chain phosphatase induces increased activation of myosin II, thereby resulting in the cross-linking of myosin II into actin filaments and increased actomyosin contraction. ROCK can also induce LIM kinase dependant-phosphorylation and subsequent inactivation of cofilin (Raftopoulou and Hall, 2004). In combination with reduced actin turnover, this ultimately leads to growth cone retraction and collapse (Raftopoulou and Hall, 2004; Sadok and Marshall, 2014) (Figure 4).
Rho GTPases regulate cytoskeleton remodelling. Rho GTPases are activated by guanine nucleotide exchange factors (GEFs) which promote the exchange of GDP for GTP. The activated Rho GTPases are then capable of interacting with downstream targets or effector proteins that can trigger remodelling of the cytoskeleton. The Rho GTPase Rac promotes lamellipodia formation, and Cdc42 promotes filopodia protrusion. Rho, however, induces actomyosin contractility. The regulatory inactivation of Rho GTPases is achieved by GTPase-activating proteins (GAPs) by stimulating the hydrolysis of GTP. It is important to realize that the pathway demonstrated here is an oversimplification of what actually occurs within the cell.

Although Rho plays a more dominant role on the chemorepulsive-facing side of the growth cone, Rho activity has also been found on the side facing an attractant where Rho stimulating actomyosin contraction is believed to help stabilize and steer the protrusion of the growth cone (Woo and Gomez, 2006). Rho has also been suggested to promote protrusion and the stabilization of actin filaments through a formin-containing protein, called mDia (mammalian diaphanous), which binds to and promotes actin polymerization (Watanabe et al., 1999). Additionally, interactions between different Rho GTPases’ signalling pathways have been observed and are believed to be involved in regulating the local activation of Rho GTPases, as well as the downstream mechanisms involved in growth cone steering and protrusion.
1.09 Retinoid Signalling in *Lymnaea stagnalis*

Retinoic acid and its various signalling components have been detected in the CNS of the invertebrate, *Lymnaea stagnalis*. Evidence from high performance liquid chromatography and mass spectrometry experiments has identified that the CNS of adult *Lymnaea* contains both retinoic acid isomers, 9-cis and all-trans retinoic acid, at relatively similar concentrations (Dmetrichuk *et al*., 2008). Both retinoic acid isomers have been shown to induce and enhance neurite outgrowth as well as induce positive growth cone turning in *Lymnaea* neurons (Dmetrichuk *et al*., 2006, 2008; Rand, 2012).

Additionally, findings have strongly suggested a role for both retinoid receptors, RXR and RAR, in non-chordate development. *Lymnaea* RXR (*LymRXR*) and the putative RAR (*LymRAR*) were shown to be expressed in the developing embryo (Carter *et al*., 2010, 2015), and the normal development of the embryo was altered by the presence of various RXR and RAR agonists or antagonists (Carter *et al*., 2010; 2015). Disrupted eye and shell development was observed in the presence of the RARβ selective antagonist, LE135, and pan-RAR antagonist, LE540 (Carter *et al*., 2015). Similar deformities were observed following the treatment of embryos with the RXR pan agonist, PA024 (Carter *et al*., 2010).

Interestingly, an increase in *LymRXR* and *LymRAR* mRNA expression was found throughout embryonic development and at stages corresponding with development of the nervous system (Carter *et al*., 2010; 2015).

In addition to demonstrating that transected *Lymnaea* growth cones could respond to RA in the absence of their cell bodies, it was also shown that the retinoid receptors were present in the growth cones (Carter *et al*., 2010; 2015). In particular, Carter *et al*. (2010) showed a non-nuclear distribution of *LymRXR* and demonstrated that the RXR agonist PA024 induced growth cone turning of both intact and isolated neurites. Furthermore, an
RXR antagonist (HX531) blocked the guidance of *Lymnaea* growth cones towards both 9-cis and all-trans retinoic acid (Rand, 2012). *Lym*RAR was also identified in regenerating neurites and growth cones (Rand, 2012) and interestingly, immunoreactivity for *Lym*RAR was found in both the cytoplasmic and membrane compartments, but not in the nucleus of adult neurons (Carter *et al.*, 2015). The RAR pan-antagonist LE540 was also shown to block both all-trans and 9-cis retinoic acid-induced chemoattraction of *Lymnaea* growth cones (Rand, 2012). Collectively, these data establish the presence and role of retinoic acid and its receptors in the developing and regenerating CNS of *Lymnaea*. It is currently hypothesized that retinoic acid binds to its receptors localized in the growth cones to mediate non-genomic signaling to induce growth cone turning. Though it is known that this process requires both calcium influx and local protein synthesis (Farrar *et al.*, 2009), the downstream effectors have not yet been identified. These may however include Rho GTPases, though this has not yet been tested in *Lymnaea* or in any other species.

**1.10 Retinoid Receptors are Conserved Between Vertebrates and Invertebrates**

Retinoic acid’s classic mode of action involves signalling through the nuclear RARs and RXRs. At least three classes of each receptor have been identified (α, β, and γ) in the human, rat, and mouse (Rosewicz *et al.*, 1995; Ulven *et al.*, 1998; Zelent *et al.*, 1998; Mangelsdorf *et al.*, 1992). In vertebrates, RXRs have been found to almost exclusively bind 9-cis retinoic acid (Heyman *et al.*, 1992), whereas RARs can be activated by both all-trans and 9-cis retinoic acid (Allenby *et al.*, 1994). RXRs are the predominant invertebrate retinoid receptors and have been shown to bind 9-cis retinoic acid in the jellyfish *Tripedalia cystophora* (Kostrouch *et al.*, 1998), fiddler crab *Uca pugilator* (Hopkins *et al.*, 2008), as well as the locust *Locusta migratoria* (Nowickj *et al.*, 2008). Interestingly, unlike the
vertebrate RXRs, the locust RXR also binds all-*trans* retinoic acid with a similar affinity to 9-*cis* retinoic acid (Nowickyj *et. al.* 2008). Moreover, all-*trans* retinoic acid was found to upregulate the expression of RXR mRNA in the marine sponge *Suberites domuncula* (Wiens *et. al.*, 2003). These findings suggest that the binding affinities of retinoid receptors may differ between vertebrates and invertebrates. In *Lymnaea stagnalis*, the LymRXR has also been cloned and has a relatively high overall homology to vertebrate (rat and human) RXRα (80%) (Carter *et. al.*, 2010). The LymRXR’s predicted ligand-binding domain shares ~81% amino acid identity with rat RXRα, and its predicted DNA-binding domain shares ~89% amino acid identity. The LymRXR also shares approximately 97% amino acid identity with the RXR of the mollusc * Biomphalaria glabrata*, which is known to bind 9-*cis* retinoic acid (Bouton *et. al.*, 2005). Although it has not yet been determined whether LymRXR binds either ligand, these findings suggested that the LymRXR will likely bind to at least 9-*cis* retinoic acid.

Non-chordate invertebrates were thought to only possess RXRs (Fujiwara and Kawamura, 2003). However, genome screening predicted the presence of an ancestral RAR in the annelid, *Capitella capitata*, and in the mollusc, *Lottia gigantean* (Albalat and Canestro, 2009). The first full-length non-chordate invertebrate RAR was cloned from *Lymnaea stagnalis* (LymRAR) (Genbank Accession # GU932671; Carter *et. al.*, 2015). However, LymRAR was found to share only 55% sequence similarity with *Homo sapiens* RARβ. LymRAR shares 85% amino acid identity with the DNA-binding domain of human RARβ, and its predicted ligand binding domain shares 58% amino acid identity with that of human RARβ. RARs have also been cloned from two other molluscs, *Thais clavigera* (Urushitani *et. al.*, 2013) and *Nucella lapillus* (Gutierrez-Mazariegos *et. al.*, 2014). Interestingly, the *Nucella lapillus* RAR is reported to not bind all-*trans* or 9-*cis* retinoic acid (Gutierrez-Mazariegos *et. al.*).
Additionally, the mollusc *Thais clavigera* RAR (TcRAR) showed no transcriptional activity in response to all-trans retinoic acid (Urushitani et al., 2013). However, any potential nongenomic activity of TcRAR would not have been detected by these transcriptional assays. Nonetheless, these results could suggest that, even though molluscs might possess the retinoic acid signalling machinery, this signalling pathway (at least the RAR) might be functioning differently compared to vertebrates. However, the ligand binding domain of TcRAR shares only 66% similarity with LymRAR (Urushitani et al., 2013), suggesting that binding affinities of LymRAR may differ compared to TcRAR (and/or *N. lapillus* RAR). It is not yet known whether LymRAR is capable of binding either all-trans or 9-cis retinoic acid or whether it may act as a potential heterodimerization partner with RXR.

### 1.11 *Lymnaea stagnalis* as a Model to Study the Role of Retinoic Acid in Regeneration

Although the adult mammalian CNS fails to regenerate, some lower vertebrates (Prince and Carlone, 2003; Dmetrichuk et al., 2005) as well as many adult invertebrates (Syed et al., 1990; Dmetrichuk et al., 2006), such as the mollusc *Lymnaea stagnalis*, have retained the ability to regenerate CNS neurons. Consequently, *Lymnaea stagnalis* was chosen as the model system for my research as it has been used in studying the effects of retinoic acid on neurite outgrowth and growth cone guidance (Dmetrichuk et al., 2006, 2008; Farrar et al., 2009; Rand, 2012). *Lymnaea* possesses a relatively simplistic nervous system in which single neurons are identifiable. Many neurons on the dorsal surface of the CNS have well characterized morphology and function, and can be successfully cultured from this animal. These molluscan neurons display regeneration *in vitro* (Syed et al., 1990) as well as *in vivo* (Syed et al., 1992). *In vitro*, large growth cones can be observed at the tips of regenerative
neurite processes, making it possible to easily manipulate and observe changes in their morphology and behaviour.

1.12 A Novel Preparation: The Floating Lymnaea CNS

Recently, a novel phenomenon has been identified in our lab, in which neurite outgrowth can be observed from cut nerves of Lymnaea CNS “floating” on the surface of culture medium. J. Simmons first identified this phenomenon and found that the nerves of the CNS must float in order to exhibit outgrowth (Figure 5; unpublished observations). If the CNS sunk to the bottom of the dish, they did not exhibit outgrowth. This suggested that increased levels of ambient oxygen at the surface and/or surface tension may play a role in the outgrowth from these floating nerves. However, previous studies conducted by D. Britt, G. Tattersall, and G. Spencer (unpublished findings) showed a greater extent of outgrowth from floating nerves of the Lymnaea CNS at lower ambient oxygen levels, suggesting this not to be the case. We are yet to investigate the role that surface tension may play in this phenomenon. Although we are still unsure of the mechanism by which the floating nerves of the intact Lymnaea CNS exhibit outgrowth, this could still prove to be a promising new model system in which to study the regenerative behaviour of invertebrate neurons.
Figure 5. Neurite outgrowth from a floating *Lymnaea* nerve. Neurites processes observed from a cut nerve ending of a *Lymnaea* CNS “floating” on the surface of Defined Medium (DM) after 72 hours. Scale bar= 60 μm.

Subsequently, (*unpublished*) studies conducted using this preparation found that the length of outgrowth from floating CNS continuously increased up to at least 5 days after *Lymnaea* CNS were extracted and floated on culture medium (Karolak, 2014; Racey, 2015). Studies also examined the effects of retinoids on this neurite outgrowth, and preliminary studies indicated that the effects were different than previously found in cultured neurons. *All-trans* retinoic acid did not induce a significant increase in the extent of neurite outgrowth from the floating CNS, when compared to the vehicle dimethyl sulfoxide (DMSO) (Karolak, 2014). However, *9-cis* retinoic acid was found to induce some trophic effects on the neurite outgrowth from the floating CNS (Racey, 2015). At higher concentrations (10⁻⁶ M), *9-cis* retinoic acid increased the number of nerves showing regeneration as well as increased the length of neurite outgrowth (Racey, 2015). It is surprising that the different retinoid isomers may affect neurite outgrowth in this model differently than previously shown in cultured cells. However, these previous studies examining the effect of the two isomers on the floating CNS were not run in parallel, and even used different concentrations of retinoids. A more
direct comparison of the abilities of the two isomers to enhance neurite outgrowth is therefore required. There are several advantages to using this model preparation to study neurite outgrowth, including the ability to observe large-scale changes, which may more closely mimic an in vivo response.

1.13 Thesis Objectives

The main aims of this thesis were twofold. The first aim was to examine the use of the floating CNS as a potential model with which to examine the effects of retinoic acid on neurite outgrowth in *Lymnaea*. Based on previous findings from cultured *Lymnaea* neurons (Dmetrichuk *et. al.* 2006; 2008), we hypothesized that retinoid isomers will enhance neurite outgrowth from the floating CNS. Furthermore, since neurites extending from floating *Lymnaea* CNS have been found to grow towards neighboring floating nerve fragments, we hypothesized that growth cones at the tip of these floating neurites would sense guidance cues and turn towards a local source of retinoic acid.

Secondly, we sought to investigate the intracellular signalling pathways involved in retinoic acid-mediated growth cone guidance. In particular, we focused on examining the effects of different retinoid receptor agonists, as well as studying the role of the Rho GTPase Rac in retinoic acid-mediated growth cone guidance. Based on previous investigations with retinoid receptor antagonists (Rand, 2012; Carter *et. al.*, 2015), we hypothesized that retinoid receptors are involved in growth cone turning towards retinoic acid. We therefore predicted that the retinoid receptor agonists would mimic the growth cone turning induced by retinoic acid. Also, since Rac has been shown to mediate growth cone attraction in response to other chemoattractants (Li *et. al.*, 2002; Yuan *et. al.*, 2003), we also hypothesized that Rac would be required for growth cone turning in response to retinoids.
Chapter 2:

The Effect of Retinoids on Neuronal Outgrowth from the Novel Floating Central Nervous System Preparation
2.01 Introduction

Retinoic acid synthesis and signalling has been shown to be up-regulated following injury in several species, such as axolotls (Maden, 1982; 1983), newts (Viviano et. al., 1995), and following sciatic nerve crush injury in adult rats (Zhelyaznik et. al., 2003). Moreover, disruption of the retinoic acid pathway has been shown to disrupt regeneration in numerous species (Maden et. al., 1982; Dmetrichuk et. al., 2005). Retinoic acid has also been found to induce and enhance neurite outgrowth in various model systems including newt spinal cord explants (Dmetrichuk et. al., 2005) and in Lymnaea stagnalis (Dmetrichuk et. al., 2006). Retinoic acid has also been shown to be involved in directed neurite outgrowth from regenerating cultured cells (Prince and Carlone, 2003; Dmetrichuk et. al., 2005). Altogether, these findings have suggested a crucial role for retinoic acid in neural regeneration.

Two biologically active isomers of retinoic acid are all-trans retinoic acid and 9-cis retinoic acid. Some studies have suggested that these two isomers act in a similar manner. Pedersen et. al. (1995) identified that both isomers increased the choline acetyltransferase activity and the intracellular levels of acetylcholine in a murine septal cell line. All-trans and 9-cis retinoic acid also induced similar inhibition of proliferation of human neuroblastoma cells (Plum and Clagett-Dame, 1995). On the other hand, other studies have demonstrated that all-trans and 9-cis retinoic acid may also act differently. In a study conducted by Han et. al. (1995), 9-cis retinoic acid was found to be more potent than all-trans retinoic acid in its antiproliferative and differentiation activity in human neuroblastoma cells, and both isomers were found to exert contrary effects on the production of extracellular matrix proteins and the proliferation of activated hepatic stellate cells from rats (Hellemans et. al., 1999). Similarly, in Lymnaea stagnalis, all-trans and 9-cis retinoic acid can have differing effects on the electric properties and intracellular calcium levels of cultured neurons. In particular, all-trans
retinoic acid was found to be more potent in inducing abnormal firing and reducing intracellular calcium levels than 9-cis retinoic acid (Vesprini and Spencer, 2014; Vesprini et al., 2015). However, both of these isomers can induce neurite outgrowth from cultured *Lymnaea* neurons (to a similar extent), and both isomers can induce growth cone turning (Dmetrichuk et al., 2006; 2008). These culture studies thus suggest that all-trans and 9-cis retinoic acid exert similar neurotrophic and tropic effects in the mollusc *Lymnaea*.

Previous research suggested that growth cone migration and axon elongation relies on adhesive contacts with solid substrates (Gomez and Letourneau, 2014). However, a novel phenomenon has been identified since, in which neurite outgrowth can be observed from the *Lymnaea* CNS “floating” on the surface of Defined Medium, i.e. in the absence of substrate adhesion. Moreover, neurites from the *Lymnaea* CNS have been found to grow towards floating nerve fragments in their vicinity, suggesting these growth cones may be capable of axon guidance in the absence of a (solid) substrate adhesion.

The main aim of this study was to examine the feasibility of this newly identified model system to investigate the effects of retinoic acid on regeneration in *Lymnaea*. In particular, we sought to determine whether all-trans and 9-cis retinoid isomers could induce neurite outgrowth from these CNS, and if so, whether the two retinoid isomers exerted similar (or differing) effects. We also aimed to determine whether growth cones of these floating neurites could demonstrate a chemotropic response to retinoic acid.

Based on previous findings from cultured *Lymnaea* nerve cells, we hypothesized that the retinoid isomers would enhance neurite outgrowth from the floating CNS in a similar manner. Furthermore, we hypothesized that growth cones at the tip of these floating neurites would be capable of sensing guidance cues and thus turn towards a local source of retinoic acid, in the absence of substrate adhesion.
2.02 Materials and Methods

Animals:

The pond snails, *Lymnaea stagnalis*, were reared and housed in open air tanks containing aerated water (with Instant Ocean Sea Salt added, (measurement 6g/L)) and were fed with romaine lettuce daily and fish food (Nutrafin Max Spirulina Algae Flake Food) and carrots twice a week. All animals used ranged in size from 16 to 20 mm in length.

Chemicals:

The retinoids all-trans (atRA) and 9-cis retinoic acid (9-cis RA) were obtained from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO) and diluted in the culture dishes containing the ‘floating’ whole CNS to produce a final bath concentration of either $10^{-6}$M or $5 \times 10^{-7}$M. Equivalent concentrations of DMSO added (vehicle control experiments) were 0.01% and 0.005% respectively. These retinoid concentrations were chosen as both concentrations have previously been used to examine outgrowth from the floating CNS (Racey, 2015) and $5 \times 10^{-7}$M is the estimated endogenous concentration of retinoids within the *Lymnaea* CNS (Dmetrichuk et al 2008).

Floating CNS Preparation:

*Lymnaea stagnalis* were anaesthetized (25% Listerine in saline) and their CNS were removed. The CNS were passed through a series of three 5 minute antibiotic saline washes (ABS; antibiotic saline: *Lymnaea* saline containing 225 μg/mL gentamicin (Sigma-Aldrich)).

Next, the CNS were pinned down in ABS and the outer connective tissue was removed (Figure 6). Preliminary trials that were conducted suggested that optimal outgrowth was observed when three CNS were placed per dish. Thus, three CNS were placed on the surface of 3 ml of DM in each dish (Falcon Easy Grip Petri dishes, 35x10mm). DM consists of 50%
L-15 (Leibowitz) media, 4X salts (NaCl 40mM, KCl 1.7mM, CaCl\(_2\) 4.1mM, MgCl\(_2\) 1.5mM and HEPES 10mM), Glutamine (60mg/L), D-Glucose (6mg/L), Gentamicin (25\(\mu\)g/mL), and autoclaved distilled water (24.6%); pH of 7.9.

![Image](image.png)

**Figure 6. Lymnaea CNS.** The *Lymnaea* CNS extracted and pinned out in a dissection dish in ABS. Scale bar: 1mm.

To analyze the trophic effect of atRA and 9-cis RA to induce neurite outgrowth, either retinoid or the vehicle (DMSO) were added into the culture dishes containing the floating CNS. Preliminary (*unpublished*) studies from our laboratory have demonstrated that floating *Lymnaea* CNS placed in a 3% ambient oxygen level exhibit more neurite outgrowth than at a 20% ambient oxygen level. All experiments (experimental and control) were therefore carried out in a 3% controlled oxygen environment to obtain maximal neurite outgrowth across the dishes. Regulation of the ambient oxygen levels was achieved using an oxygen regulator (Sable Systems International) kindly provided by G. Tattersall.

**Growth Cone Turning Assays:**

As described above, three *Lymnaea* CNS were extracted and placed on the surface of DM. Approximately 16 to 18 hours following plating, growth cones could be observed at the
tips of neurites extending from the floating CNS. All-trans retinoic acid was prepared in absolute ethanol (EtOH; Sigma-Aldrich) and diluted to a final concentration of 10⁻⁵M using DM (0.1% final EtOH concentration). All-trans retinoic acid was pressure-applied onto the growth cones through a pipette, using a Femtojet microinjector (Eppendorf, Canada). The pipette was placed between 400 to 500μm from the growth cone, depending on both the size of the pipette opening (range= 8-10μm) and the pressure applied. A holding pressure of 1-2hPa was used to prevent backflow into the pipette between applications. Growth cone movement was monitored and photographed throughout, using Northern Eclipse imaging software (Empix imaging, ON). Images were taken every 4-5 minutes over the course of each growth cone turning assay. Due to the slow response and sensitive nature of floating growth cones, no vehicle control experiments were conducted at this time, though numerous published findings from our lab have established that application of the vehicle alone does not induce positive growth cone turning (Dmetrichuk et. al., 2006; 2008; Farrar et. al., 2009; Rand, 2012; Rand et. al., 2017).

**Data and Statistical Analysis:**

All images were captured using an inverted Zeiss Axiovert 200 microscope, Retiga Exi camera and Northern Eclipse software (Empix Imaging Inc.). Neurite outgrowth was assessed approximately 72 hours following plating. The proportion (%) of floating nerves that exhibited neurite outgrowth in the presence of DMSO, 9-cis RA, and atRA in the bath was determined. Only floating nerves were counted, as those submerged do not exhibit outgrowth. A Chi-square test was used to determine significance. In order to determine if retinoids enhanced the length of neurites, the average of the three longest neurite processes per cut nerve was determined for only nerves that exhibited outgrowth. The mean length of
the neurites was then determined in each of the three conditions: DMSO, 9-\textit{cis} RA, and atRA. Values were compared using a Kruskal-Wallis One Way ANOVA on Ranks, and a Dunn’s \textit{post hoc} test was used to determine statistical significance. Data were deemed significant when \( p < 0.05 \).

In order to determine whether retinoids induced more outgrowth (density), the cut nerve ending with the densest neurite outgrowth from each CNS was selected and the growth coverage of a defined area was analyzed. Preliminary observations indicated that in all three conditions (DMSO, 9-\textit{cis} RA, and atRA) neurites usually grew up to a length of at least 200\( \mu \text{m} \) from the cut nerve endings. No previous recordings of outgrowth density have been made, and as such, an arbitrary circular area with a diameter slightly greater than 200\( \mu \text{m} \) (207.8\( \mu \text{m} \)) was chosen to analyze neurite coverage. This circular area was identical in size for each measurement. It was drawn in ImageJ and placed at the cut nerve endings, such that the center of the circle aligned with the center of the cut nerve ending (Figure 7). The neurite outgrowth was highlighted in ImageJ and the proportion (\%) of space occupied by this neurite outgrowth (neurite coverage) within the defined area (135620 \( \mu \text{m}^2 \)) was determined (Figure 7 and Appendix 5). Values for neurite coverage in all conditions DMSO, 9-\textit{cis} RA, and atRA were compared using a Kruskal-Wallis One Way ANOVA on Ranks, and a Dunn’s \textit{post hoc} test was used to determine statistical significance. Data were deemed significant when \( p < 0.05 \).
Figure 7. Analysis of the proportion of neurite outgrowth coverage within a defined area. Representative images of neurite outgrowth from a cut nerve ending of a *Lymnaea* CNS “floating” on the surface of DM (A). The proportion (%) of neurite outgrowth coverage was determined within a defined area of 135620 \( \mu m^2 \) (indicated by circle) from the cut nerve ending using ImageJ software, as demonstrated in B. The dashed arrows indicate aligning the center of the circle to the center of the cut ending (B). Scale bar= 60\( \mu \)m (A).

For the growth cone turning assays, the individual growth cone turning angles were determined by measuring the angle between the growth cone's initial trajectory and the final angle of turning observed over the course of the experiment (Figure 8). Growth cones were monitored for a minimum of 1 hour (as done previously in cultured neurons; Farrar *et. al.*, 2009; Carter *et. al.*, 2010) to a maximum of 4 hours (additional time was required as neurites appeared to grow slower than in cultured neurons moving on a substrate). An attractive turning response consisted of the growth cone turning towards the pipette and was deemed as a positive angle.
Figure 8. Measurement of the turning angle of floating growth cones in response to atRA.
(A) Representative image of floating growth cones extending from a Lymnaea CNS. The black arrow points to the growth cone analyzed in this figure. The red line at the top of the image and the dashed white line indicate the growth cone’s initial trajectory, before locally applying atRA. (B) The floating growth cone’s turning response to a local source of atRA (indicated by a pipette on the right side of the image) was measured. The initial trajectory of the growth cone, marked in (A), was superimposed onto the final recorded image of the growth cone turn (B). The red line indicates no change in the angle of the neurite near the cut nerve ending. The dashed white line indicates a slight change in the angle of the neurite immediately preceding the growth cone studied, and this has also been observed in growth cone studies performed in cell culture. The dotted white arrow indicates the final turning response recorded for the growth cone. The asterisk represents the angle measured as the turning response for this growth cone. Times are given in minutes. Scale bar = 25μm.
2.03 Results

The Effect of Retinoids on Neurite Outgrowth

In *Lymnaea stagnalis*, it has been demonstrated that atRA and 9-cis RA induce similar trophic effects on cultured nerve cells (Dmetrichuk *et. al.*, 2006; 2008). However, a difference in the effects of these retinoid isomers has been observed when examining the electric properties and intracellular calcium levels of cultured *Lymnaea* neurons (Vesprini and Spencer, 2014; Vesprini *et. al.*, 2015). Since our lab has recently determined that neurite outgrowth can be observed from the *Lymnaea* CNS placed on the surface of DM, we sought to investigate the effects of these retinoid isomers on the extent of neurite outgrowth and to determine whether the effects would be similar between atRA and 9-cis RA isomers.

The extent of neurite outgrowth from floating *Lymnaea* CNS, in higher ($10^{-6}$M) and lower ($5x10^{-7}$M) concentrations of retinoids and equivalent concentrations of DMSO (0.01% and 0.005%, respectively) was analyzed. Representative images depicting neurite outgrowth observed from cut nerve endings of *Lymnaea* CNS floating on the surface of DM with DMSO, 9-cis RA, or atRA in the bath are shown in Figure 9. The proportion (%) of floating nerves that exhibited outgrowth of neurites after 72 hours in each treatment was determined and a Chi-square test showed no significant effect of treatment at higher concentrations of retinoids ($10^{-6}$M: Chi-square= 0.48, df=2; p = 0.787; Figure 10 A). However, at lower concentrations ($5x10^{-7}$M), a significant effect of treatment was observed (Chi-square= 13.705, df= 2; p = 0.001). The proportion of floating nerves that exhibited outgrowth was significantly less in atRA compared to both 9-cis RA (p=0.004) and DMSO (p=0.006; Figure 10 B).
Figure 9. Neuronal outgrowth from floating CNS in DMSO, 9-cis RA, and atRA. Two different representative images for each treatment, taken at 72 hours, depicting the variation in the extent of neurite outgrowth observed from cut nerve endings emerging from floating Lymnaea CNS in: (A) DMSO, (B) 9-cis RA, or (C) atRA. Scale bars= 60 μm.
Figure 10. Fewer nerves exhibit neurite outgrowth in atRA at lower concentrations.
Summary graphs showing the proportion of floating nerves that exhibited outgrowth after 72 hours in the presence of 9-cis RA and atRA at a concentration of $10^{-6}$M (A) and $5 \times 10^{-7}$M (B), and equivalent concentrations of the vehicle DMSO (0.01% (A) and 0.005% (B), respectively). Statistical analysis used a Chi-square test and determined that at higher concentrations of $10^{-6}$M, there was no significant effect of treatment (Chi-square= 0.48, df=2; $p = 0.787$). However, at lower concentrations of $5 \times 10^{-7}$M, a significant effect of treatment was observed (Chi-square= 13.705, df= 2; $p = 0.001$). The proportion of floating nerves that exhibited outgrowth was significantly less in atRA compared to both 9-cis RA ($p=0.004$) and DMSO ($p=0.006$). N values for each treatment are provided in brackets and represent the total number of floating nerves analyzed (including floating nerves that did not exhibit outgrowth).
The maximal length of outgrowth from all cut nerves exhibiting outgrowth in each treatment was also determined. A Kruskal-Wallis One Way ANOVA on Ranks found no significant difference in the maximal length of neurites in each treatment at both higher concentrations of retinoids \( (10^{-6}\text{M}: H = 0.585, \text{df}=2; p=0.746; \text{Figure 1 A}) \) and lower concentrations \( (5 \times 10^{-7}\text{M}: H = 3.345, \text{df}=2; p=0.188; \text{Figure 1 B}) \). All three treatments produced neurites of equivalent lengths.

For the above two parameters measured, more than one cut nerve emanating from one CNS was analyzed. This gave rise to the consideration that the extent of neurite outgrowth from one individual cut nerve might depend on which CNS it originated from, and may be influenced by other nerves emanating from the same CNS. Though it is currently unknown whether the outgrowth from one nerve can directly affect the outgrowth from another, the proportion (\%) of floating nerves that exhibited neurite outgrowth and the maximal length of outgrowth observed were therefore re-analyzed keeping the “identity” of the nerve (which CNS it emerged from), a consideration in the analysis. These additional data, analyzed using R software (© The R Foundation), are presented in the appendix (Appendix 1 and 2). Overall, the findings of these additional analyses were similar to those mentioned above (which assumed independence of all observations).
Figure 11. Retinoids do not increase the mean length of neurite outgrowth. Summary graphs showing the maximal length of neurite outgrowth observed in 9-cis RA and atRA at a concentration of $10^{-6}$M (A) and $5 \times 10^{-7}$M (B), and in DMSO (at equivalent concentrations of 0.01% (A) and 0.005% (B), respectively). Error bars represent the standard error of the mean (S.E.M.). Statistical analysis was performed using a Kruskal-Wallis One Way ANOVA on Ranks. No significant difference was found amongst treatments, at higher ($10^{-6}$M: $H = 0.585$, df=2; $p=0.746$) or lower concentrations ($5 \times 10^{-7}$M: $H = 3.345$, df=2; $p=0.188$). N values for each treatment are provided in brackets and represent the total number of nerves analyzed. Only nerves that exhibited outgrowth were analyzed.
Though the retinoid treatments did not induce longer neurite outgrowth, it was possible that they affected the “density” of outgrowth emerging from the cut nerves. The proportion (%) of space covered by neurite outgrowth within a defined area was thus also determined. A Kruskal-Wallis One Way ANOVA on Ranks found no significant difference in the neurite coverage of the defined area between vehicle control treatment and retinoids at the higher concentration (10⁻⁶M: H = 0.645, df=2; p = 0.724; Figure 12 A). However, at lower retinoid concentrations (5x10⁻⁷M), a significant effect of treatment was observed (H= 9.456, df= 2; p=0.05). A Dunn’s post-hoc test revealed that a significantly higher neurite coverage of the defined area occurred in the presence of 9-cis RA compared to atRA (p=0.009; Figure 12 B).

Overall, these data indicate that at lower concentrations (5x10⁻⁷M), 9-cis RA appeared to promote more outgrowth from the Lymnaea CNS, than atRA. These effects, however, were not replicated at higher concentrations (10⁻⁶M), where there was no significant effect of retinoids on the extent of neurite outgrowth from the Lymnaea CNS.
Figure 12. 9-cis RA induces more neurite coverage than atRA. Summary graph showing the mean neurite coverage (% of a defined area) in the presence of 9-cis RA and atRA at a concentration of $10^{-6}$M (A) and $5 \times 10^{-7}$M (B), and in DMSO (at equivalent concentrations of 0.01% (A) and 0.005% (B), respectively). Error bars represent the standard error of the mean (S.E.M.). The cut nerve ending with the “densest” neurite outgrowth on each CNS was selected for growth analysis. A Kruskal-Wallis One Way ANOVA on Ranks determined that at higher concentrations there was no significant effect of treatment ($10^{-6}$M: $H = 0.645$, df=2; $p = 0.724$). However, at lower concentrations, a significant effect of treatment was observed ($5 \times 10^{-7}$M: $H = 9.456$, df=2; $p=0.05$) and a Dunn’s post-hoc test determined that 9-cis RA induced significantly higher neurite coverage than atRA (*$p=0.009$). N values for each treatment are provided in brackets and represent the total number of nerves analyzed.
atRA Induced Growth Cone Turning of Floating Neurites

Whilst examining neurite outgrowth from floating CNS, it was noted that neurites occasionally grew towards floating nerve fragments found in their vicinity, suggesting that they are capable of sensing and responding to chemotropic guidance cues. Previous findings have determined that retinoic acid can induce a chemotropic effect on growth cones of cultured Lymnaea cells (Dmetrichuk et. al., 2006; 2008). We therefore conducted a pilot study to determine whether growth cones on these floating neurites could indeed sense chemical guidance cues, by testing whether they would turn towards a local source of retinoic acid. A local gradient of atRA ($10^{-5}$ M) was focally applied onto growth cones at the tips of floating neurite processes. The growth cones were found to turn toward the source (pipette) of retinoic acid with a mean turning angle of $25.3 \pm 6.5^\circ$ (n=9; Figure 13 A-D). A graph showing the final turning angle of each individual growth cone in response to atRA is shown in Figure 13 E. Individual growth cone turns toward atRA are depicted as a positive turning angle in the graphs. Overall, the data from these pilot studies support the hypothesis that the floating growth cones (in the absence of solid substrate adhesion) are capable of responding to guidance cues.
Figure 13. Floating growth cones are attracted to a gradient of atRA. (A-D) Representative images depicting the turning responses of growth cones (indicated by arrows) to a local gradient of atRA. The pipette tip is shown in the lower right hand corner. E. A graph showing the individual final turning angles toward atRA with each bar representing one growth cone (n=9). Times are given in minutes. Scale bar = 25μm.
2.04 Discussion

The Effect of Retinoids on Neuronal Outgrowth from the Novel CNS Preparation

It has generally been established that neurite outgrowth and growth cone motility are dependent on the adhesion of cells to a solid substrate (Suter and Forscher, 2000; Koch et al., 2012). However, our lab has recently shown that neurite outgrowth can also occur from Lymnaea CNS floating on the surface of DM (J. Simmons, unpublished findings). Since only cut nerves of the Lymnaea CNS that were floating exhibited neurite outgrowth, we had initially speculated that increased levels of ambient oxygen at the surface may play a role in the induction of this outgrowth. However, previous studies conducted by D. Britt, G. Tattersall, and G. Spencer (unpublished findings) showed a greater extent of outgrowth from floating nerves of the Lymnaea CNS at lower ambient oxygen levels (e.g. 3% and 5% oxygen levels compared to 20%), suggesting that increased oxygen levels are not responsible for this outgrowth. Another possibility that we have not yet examined is that surface tension of the fluid plays a role in inducing outgrowth. Although we are still unsure of the mechanism by which the floating cut nerves of the intact Lymnaea CNS exhibit this increased neurite outgrowth, this new model could prove promising to study the regenerative behaviour of invertebrate neurons, as the neurite outgrowth is easily monitored and measured over time.

One molecule that has been implicated in regeneration in both vertebrates and invertebrates is retinoic acid. For example, a role for retinoic acid in inducing and enhancing neurite outgrowth has been shown in newt spinal cord tissue (Dmetrichuk et al., 2005) and embryonic chick dorsal root ganglia neurons (Maden et. al., 1998). Previous studies in Lymnaea have shown that the retinoid isomers 9-cis RA and atRA induce similar trophic effects on neurite outgrowth from cultured motorneurons (Dmetrichuk 2006; 2008). Moreover, 9-cis RA and atRA were both shown to induce positive turning of Lymnaea
growth cones in culture (Dmetrichuk et al., 2006; 2008). Consequently, my main aim was to determine the effect of these retinoid isomers on the extent of neurite outgrowth and the growth cone turning behaviour of floating neurites, emerging from floating CNS.

Preliminary studies using this preparation have previously found no significant increase in the extent of neurite outgrowth from the floating CNS in the presence of atRA, compared to the vehicle DMSO (Karolak, 2014). However, the floating CNS were only treated to lower concentrations of atRA ($5 \times 10^{-7}$ M) and the threshold concentration needed to see an effect may have been higher. On the other hand, some significant effects were previously shown for 9-cis RA at a higher concentration of $10^{-6}$ M, compared to the vehicle DMSO (Racey, 2015). These effects included an increase in the proportion of nerves exhibiting neurite outgrowth and an increase in the length of outgrowth at some timepoints (but not at others).

In an attempt to resolve the ambiguity of the preliminary results previously obtained, my first aim was to directly compare the effects of the two retinoid isomers, atRA and 9-cis RA, under similar conditions and at the same time points. My data suggested an ability of 9-cis RA to enhance neurite outgrowth but the effects were not consistent across different concentrations tested. However, it was apparent that 9-cis RA was more likely to enhance neurite outgrowth compared to atRA, and these findings were consistent with preliminary data obtained by Karolak (2014) and Racey (2015). It is unclear why 9-cis RA showed significant differences to atRA at only the lower concentration, but one possibility is that effect of the retinoid isomers on the extent of neurite outgrowth may have been compounded by negative effects of the vehicle (DMSO) at higher concentrations. At the higher concentrations of DMSO (0.01%), my data showed a reduction in the proportion (%) of floating nerves that exhibited outgrowth, the maximal length of outgrowth, and the “density”
of outgrowth, compared to lower concentrations of DMSO (0.005%). If neurite outgrowth is dependent on surface tension, it is feasible that DMSO may have altered this property and ultimately interfered with the ability of the nerves to exhibit outgrowth. However, further research will be needed to test this hypothesis, as well as to more closely examine possible differences between the two retinoid isomers.

Until now, previous findings have been unable to demonstrate that retinoic acid consistently enhances neurite outgrowth from the floating Lymnaea CNS. One possible reason for this might be that the CNS is secreting other trophic factors, whose effects may be manifested to a greater extent than those of exogenously applied retinoic acid, thus masking its effects. Indeed, previous studies in Helisoma support the notion that trophic factors are released from the isolated CNS (Wong et al., 1981). This possibility might help explain the lack of significant effects of the retinoid isomers on the length of neurite outgrowth from the CNS, compared to the vehicle. These data also appear contrary to the neurite-inducing effects previously observed by the two isomers on cultured neurons. However, it should be noted that previous studies examined the effects of the retinoids on cultured Lymnaea neurons in the absence of any other trophic factors (Dmetrichuk et al., 2008).

A lack of a consistent effect of retinoic acid in enhancing neurite outgrowth from the floating CNS may also be due to increased metabolism of retinoic acid by the CNS (compared to the metabolism of retinoic acid by a small number of individual neurons in culture). It is very likely that the retinoic acid was degraded or metabolised much faster by the CNS than by cultured neurons (Christie et al., 2008). In the floating CNS experiments, the retinoic acid was added only once, on the first day, to a dish containing three floating CNS which would likely metabolize retinoic acid relatively quickly, thereby reducing its trophic effects. This would not occur to the same extent with cultured cells. Any further
research that intends to continue examining the effects of retinoids on neurite outgrowth from the floating CNS will likely need to refine the experimental protocols in order to obtain more consistent effects.

Nonetheless, the results found in my study are intriguing as they suggest that retinoid isomers may induce different effects on neurite outgrowth from the floating *Lymnaea* CNS. This differs to what was previously observed in cultured *Lymnaea* neurons where it was previously shown that 9-cis RA and atRA exerted similar trophic effects (Dmetrichuk 2006; 2008). However, it should be noted that not all effects of these retinoids are similar in *Lymnaea*, as previous studies have also shown different effects of all-trans and 9-cis RA on their ability to change both firing properties and intracellular calcium levels (Vesprini and Spencer, 2014; Vesprini *et. al.*, 2015). Since neuronal activity and intracellular calcium are well known to affect both gene transcription and neurite outgrowth, it is quite possible that these cellular properties may be involved in the differing effects of the isomers seen in this study.

**The Effect of atRA on the Growth Cone Turning Behaviour of Floating Neurites**

In addition to examining the effect of retinoid isomers on the extent of neurite outgrowth on the floating CNS, we also sought to investigate the growth cone turning behaviour of these floating neurites. Previous research suggested that growth cone migration and axon elongation relies on adhesive contact with a solid substrate in order to occur (Gomez and Letourneau, 2014). However, we have clearly demonstrated that neurite outgrowth can also occur from floating *Lymnaea* CNS in the absence of any adhesive contacts with a solid substrate. Anecdotal evidence and observations from previous preliminary studies in the lab have also suggested that these floating neurites can grow
towards neighboring floating nerve fragments. This gives rise to the possibility that growth cones on these floating neurites might be able to sense and respond to guidance cues in the absence of (solid) substrate adhesion.

My second aim was thus to conduct a pilot study to investigate whether the floating growth cones could respond to a guidance cue, and indeed demonstrated that the growth cones responded by turning toward a focal source of atRA. However, both neurite extension and growth cone turning was found to occur very slowly in these floating preparations; in most instances, it took between 1 and 4 hours to obtain growth cone turning responses. It is possible that the growth cone response occurred more slowly (than that of cultured growth cones), either as a result of the slower rate of outgrowth or due to the absence of a solid substrate. Indeed, previous studies have suggested that the speed of growth cone advancement might rely on the strength of adhesiveness to a substrate (Letourneau, 1975; Gomez and Letourneau, 2014). Moreover, the floating growth cones were found to be extremely sensitive to agitation of the surface of the solution (such as occurred by the insertion of the pipette and occasionally during application of the retinoic acid). However, this might further support a role for surface tension in the outgrowth from these floating CNS.

As a result of the increased “sensitivity” of the growth cones, we determined that this preparation was likely not a feasible model system for further examination of the chemotropic effects of retinoic acid (or for further elucidation of the transduction pathways involved in growth cone turning responses). Furthermore, due to the slow response and sensitive nature of the floating growth cones, no vehicle control experiments were conducted at this time. However, it should be noted that previous findings from our lab have consistently shown that application of any vehicle (either EtOH or DMSO) does not induce
any positive growth cone turning (Dmetrichuk et al., 2006; 2008; Farrar et al., 2009; Carter et al., 2010; 2015; Rand, 2012; Rand et al., 2017). However, these findings were in cultured neurons and so we cannot completely rule out the possibility (though unlikely) that vehicle application would also have no effects on the floating growth cones.

Nonetheless, these experiments have provided the first preliminary evidence for a chemotropic response of floating neurites in the absence of solid substrate adhesion and further support a physiological role for atRA in axonal pathfinding. Moreover, the tropic effects of retinoic acid were only previously shown using Lymnaea motorneurons (PeA and Visceral F), but data here suggest that the other cell types may also be responsive to atRA.

Overall, in summary, a novel phenomenon has been identified in our lab in which neurite outgrowth can be observed from Lymnaea CNS floating on the surface of defined (culture) medium. Unlike previous findings from Lymnaea cells in culture, there was a lack of a consistent effect of retinoid isomers in promoting neurite outgrowth from the floating CNS. Interestingly however, findings may suggest a greater potential for 9-cis RA to enhance neurite outgrowth than atRA, but, further research will be required to verify this. Nonetheless, atRA was still able to induce a chemotropic response from growth cones of these floating neurites, in the absence of substrate adhesion. This novel finding demonstrates that, unlike previously believed, growth cones can still sense and respond to guidance cues (at least retinoic acid) in the absence of substrate adhesion.
Chapter 3:

An Investigation of the Cellular Mechanisms Involved in Retinoic-acid Mediated Growth Cone Turning
3.01 Introduction

Retinoic acid has been implicated in the development (Chen and Solursh, 1992; 1994) and regeneration of various organ systems in several species (Tsonis et al., 2002; Maden and Hind, 2003). This includes the nervous system (Maden, 1996; 1998; Dmetrichuk et al., 2005), where it can exert trophic effects to initiate and maintain neurite outgrowth (Corcoran et al., 2002; Prince and Carlone, 2003; Dmetrichuk et al., 2005). The main effects of retinoic acid are thought to occur via signalling through nuclear receptors, which include retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which then regulate gene transcription. RARs and RXRs have different binding affinities for different retinoid isomers (Heyman et al., 1992; Allenby et al., 1994), but these binding affinities appear to differ between vertebrates and invertebrates (Nowickyj et al., 2008). In the mollusc Lymnaea, both RXR (LymRXR) and the putative RAR (LymRAR) have been cloned (Carter et al., 2010; 2015), but their binding affinities and/or their ability to heterodimerize are not yet known. In addition to transcriptional activity of retinoic acid via its nuclear receptors, nongenomic effects of retinoic acid have also been described, such as regulation of homeostatic plasticity in the vertebrate hippocampus (Aoto et al., 2008; Maghsoodi et al., 2008).

Nongenomic effects of retinoic acid have also been found to underlie chemotropic effects of retinoids to induce growth cone turning in Lymnaea neurons (Farrar et al., 2009). Retinoic acid acts as a chemoattractant for vertebrate spinal cord neurons (Maden et al., 1996; 1998; Dmetrichuk et al., 2005) as well as invertebrate motoneurons (Dmetrichuk et al., 2006; 2008; Farrar et al., 2009; Rand, 2012). In Lymnaea, growth cones mechanically isolated from their cell bodies have been found to retain their capacity to respond to, and turn toward retinoic acid (Farrar et al., 2009). Studies in Lymnaea have previously shown LymRXR is present in the growth cones of cultured regenerating motoneurons (Carter et al.,
Furthermore, a RXR agonist, PA024, was able to induce growth cone turning in both intact and isolated neurites (Carter et al., 2010). Carter et al. (2015) also recently identified a non-nuclear distribution for the LymRAR in growth cones, though no studies to date have shown whether RAR agonists can mimic the chemoattractive effects of retinoic acid. In this study, we thus aimed to determine whether various RAR agonists and synthetic retinoids could mimic the effects of retinoic acid to produce growth cone turning in cultured regenerating Lymnaea neurons. Although the RXR agonist, PA024, was previously tested and found to induce growth cone turning in Lymnaea, this agonist is no longer available for use. Since there has also been limited study of this receptor to date, an alternative (commercially available) RXR agonist, SR11237, was tested to determine whether it would induce growth cone turning.

Although previous studies have provided some evidence for the role of neuritically localized retinoid receptors in the chemoattractive effects of retinoic acid, far less is known of other potential signalling components involved in this response. To date, retinoic acid-mediated growth cone turning has been shown to require both local protein synthesis and calcium influx (Farrar et al., 2009). However, the signalling cascades which link the calcium influx to regulation of the cytoskeleton are not currently known. A possible link might be the activation of Rho GTPases, which include Ras-related C3 (Rac). Rho GTPase signalling occurs downstream of activation of the growth cone membrane receptors and calcium signals, and upstream of effectors of cytoskeletal dynamics. Rho GTPases have been shown to mediate growth cone responses to a number of guidance cues in the nervous system (Yuan et al., 2002). In particular, Rac mediates growth cone attraction to netrin in mice (Li et al., 2002), and to brain-derived neurotrophic factor (BDNF) in Xenopus laevis (Yuan et al., 2003). However, whether Rho GTPases play a role in retinoic acid-induced chemoattraction
has not yet been studied. The aim of this study was thus to determine whether the Rho
GTPase Rac is involved in retinoic acid-mediated growth cone turning in regenerating
*Lymnaea* neurons.
3.02 Materials and Methods

Animals:

The pond snails, *Lymnaea stagnalis*, were reared and housed in open air tanks containing aerated filtered water. Water was supplemented with salts (Instant Ocean Sea Salt) at a concentration of 6g/L. They were fed romaine lettuce daily, and fish food (Nutrafin Max Spirulina Algae Flake Food) and carrot shavings twice a week. All animals used for cell culture experiments ranged in size from 16 to 20 mm in length.

Preparation of Poly-l-Lysine Coated Culture Dishes and *Lymnaea* Conditioned Medium (CM):

Cell culture dishes (Falcon Easy Grip Petri dishes, 35x10mm) were prepared by drilling a hole (1cm in diameter) through the bottom of the dish. A glass cover slip was attached to each dish below the drilled hole and the plates were sterilized with 95% ethanol (EtOH). Once dry, the dishes were coated overnight with poly L lysine (1mg/mL Tris buffer). The following morning, a glass pipette was used to remove the poly L lysine and the dishes were left to dry for 40 minutes. The dishes were then washed three times (15 minutes each) with distilled water and left to dry. These poly L lysine coated culture dishes were stored between +2 and 8°C and used within 7 days.

*Lymnaea* Conditioned Medium (CM) contains (unidentified) trophic factors that are required for generating outgrowth in vitro. CM was prepared by dissecting 12 *Lymnaea* CNS from animals ranging in size from 20-30 mm. These CNS were then passed through a series of eighteen washes with antibiotic saline (ABS; sterile saline containing gentamycin at 225μg/mL), for a duration of seven to ten minutes each. Next, the 12 CNS were placed in 7.5ml of Defined Medium (DM) in Sigma-coted (Sigma-Aldrich) glass dishes (Pyrex,
60x15mm) for incubation. DM consists of 50% L-15 (Leibowitz) media, 4X salts (NaCl 40mM, KCl 1.7mM, CaCl$_2$ 4.1mM, MgCl$_2$ 1.5mM and HEPES 10mM), Glutamine (60mg/L), D-Glucose (6mg/L), Gentamicin (25μg/mL), and autoclaved distilled water (24.6%); pH of 7.9. Following incubation, which lasted 4 days, Lymnaea CNS were again passed through a series of eight, seven to ten minute ABS washes, before being transferred to a fresh dish of DM for subsequent incubations (2 more in total, each for 3-4 days). CM incubated with CNS from the first 4 days was discarded as it is generally thought to contain inhibitory factors that do not support neurite outgrowth. CM from the subsequent two incubation periods were used to culture neurons and to induce neurite outgrowth.

**Cell Culture Procedures:**

Snails were anaesthetized (25% Listerine® in saline) and their CNS removed. The CNS were passed through a series of three 5 minute ABS washes. Next, the CNS were treated with trypsin (Sigma-Aldrich; 6mg in 3mL DM) for 19.5 to 22 minutes, followed by trypsin inhibitor (Sigma-Aldrich; 6mg in 3mL DM) for 10 minutes. The CNS were then pinned out in high osmolarity DM (800μL of 1M Glucose in 30mL DM) and the outer connective tissue and inner sheath surrounding the left and right Pedal ganglia were removed.
Individual Pedal A motorneurons (PeA) were chosen for these studies as they have been shown to generate extensive neurite outgrowth in cell culture (Spencer et al., 1995; Dobson et al., 2006; Farrar et al., 2009), and have been used in previous growth cone studies examining the effects of retinoic acid in *Lymnaea* (Carter et al., 2010; 2015). The PeA cells were removed from the left and right Pedal ganglia using a fire-polished glass pipette coated with Sigmacote (Sigma-Aldrich) to prevent cell adhesion. Suction was applied using a micrometer syringe (Gilmont® Instruments, 0.2 mL) to remove individual cell bodies from the CNS and these cells were then plated into poly L lysine coated culture dishes containing 2.5mL of CM and 0.5mL of DM (Figure 15). All-trans retinoic acid (atRA) was added to the culture dishes at the end of cell plating (10^{-7}M final bath concentration) to promote neurite outgrowth. Extensive outgrowth was observed within 16 to 18 hours following plating.
Chemicals:

AtRA was purchased from Sigma Aldrich. The synthetic retinoids: CH55 (which has high affinity for vertebrate RAR-α/β receptors), EC23 (an analog of atRA), and SR11237 (a selective RXR pan agonist), were obtained from Tocris Bioscience. Refer to Appendix 3 and 4 for a full description of the synthetic retinoids used in this study. To investigate the effects of synthetic retinoids on growth cone turning, atRA and all synthetic retinoids were prepared in 100% dimethyl sulfoxide (DMSO) and diluted using DM to a concentration of $10^{-5}$M (in a final concentration of 0.1% DMSO) and added to the pipette. SR11237 was also tested at $10^{-6}$M (pipette concentration), in order to compare directly with data obtained previously with another RXR pan-agonist, PA024 (Carter et. al., 2010). Vehicle control experiments used a concentration of 0.1% DMSO (in DM) in the pipette.
The Rac inhibitor, NSC23766, was obtained from Sigma Aldrich. NSC23766 was
diluted in sterile distilled water and added to the bath at least 1 hour before growth cone
turning assays, to produce a final bath concentration of 100μM (Hou et. al., 2014).

**Growth Cone Turning Assays:**

Approximately 16 to 18 hours following plating of PeA cells, cells exhibiting neurite
outgrowth and active growth cones were monitored (Figure 16) and imaged using an inverted
microscope (Zeiss Axiovert 200). Only growth cones exhibiting a steady growth trajectory
for at least 15 minutes were used.

![Figure 16: Representative example of PeA neuronal outgrowth in culture](image)

Either atRA or synthetic retinoids (CH55, EC23, or SR11237) were pressure-applied
onto the growth cones via a pipette (4-6μm in diameter, pressure 2-7 hPa), using an
Eppendorf–Femtojet. The pipette was placed between 50 and 150μm from the growth cone
(depending on the size of the pipette), and the pressure applied was adjusted accordingly. A
holding pressure of 1-2hPa was used to prevent backflow into the pipette between
applications. In order to test the role of Rho GTPases in atRA-mediated growth cone turning, the Rac inhibitor NSC23766 was bath applied. After 1 hour, the active growth cones were monitored once again to confirm that the addition of the Rac inhibitor had not disrupted neurite outgrowth. Test agents (atRA or EC23) were applied to the growth cones in the presence of the Rac inhibitor.

In order to examine the role of Rac on isolated growth cone responses, the neurites were transected from the cell body using a sharp glass electrode (Figure 17). Fifteen minutes following isolation, the transected *Lymnaea* neurites were monitored and growth cones with sustained activity were used for growth cone turning assays. A gradient of atRA (or EC23) was applied in a similar manner as described previously for intact neurites. The transected neurites were monitored throughout the entire experiment to ensure no contact was re-established with the cell body (or any adjacent neurites attached to the cell body).

**Figure 17. A neurite mechanically isolated from its cell body.** (A) An intact neurite connected to the cell body. (B) The intact neurite was severed near the cell body. (C) A complete separation of the neurite from the cell body. The cell body of the neuron was either completely removed from the dish or left in the dish (as in image C) but checked regularly to ensure the neurite remained separated from the cell body throughout the experiment. Scale bar: 15μm.
**Growth Cone Measurements:**

All images were captured using a Zeiss Axiovert 200 inverted microscope and Retiga Exi camera with QCapture Suite 2.90.1 software (Quantitative Imaging Corporation). Individual growth cone turning angles were determined by measuring the angle between the growth cone's initial trajectory and the maximum angle of turning observed over a time course of 50 to 60 minutes. Images were taken every 4-5 minutes over the course of each growth cone turning assay. An attractive turning response consisted of the growth cone turning towards the pipette and is indicated in the data with a positive angle. A negative response consisted of the growth cone turning away from the pipette and is indicated in the data with a negative angle.

When examining the effect of the Rho GTPase, the length of neurite extension was also measured, using Northern Eclipse imaging software (Empix imaging, ON). Growth cone advancement was measured as the distance travelled by the tip of the growth cone at the beginning of the assay to its final location at the end of the experiment. An increase in the length of the neurite by the end of the experiment produced a positive value whereas neurite retraction produced a negative value.

**Data and Statistical Analysis:**

All statistical analyses were performed using SigmaStat software. Growth cone turning angles and length of extension measurements induced by each retinoid were expressed as mean ± standard error of the mean (S.E.M.). Comparisons of turning angles and length of extension measurements for each experiment were performed using a Two Way ANOVA with a Holm–Bonferroni Sequential Correction post-hoc test (unless stated otherwise).
3.03 Results

The effects of synthetic retinoids on growth cone turning in the pond snail, *Lymnaea stagnalis*

We have previously shown the presence of both LymRAR and LymRXR in *Lymnaea* growth cones (Carter *et. al.*, 2010, 2015). However we have only previously shown that one synthetic retinoid, the RXR pan agonist PA024, can mimic retinoic-acid induced growth cone turning. Unfortunately, this agonist is no longer available for use. Hence, the first aim was to examine the effects of various commercially available RAR and RXR agonists on their ability to induce growth cone turning in *Lymnaea* neurons. These studies will provide insight into which synthetic agonists most closely mimic the effects of retinoic acid, as well as providing further insight into the role of either LymRAR and LymRXR in retinoic-acid mediated chemotropic effects.

Growth cones turn towards a gradient of atRA

Before testing the effects of various commercially available retinoid agonists on *Lymnaea* growth cone turning, it was necessary to ensure repeatability of previous findings, in that cultured PeA neurites were attracted to a gradient of atRA (Farrar *et. al.*, 2009). Thus, a local gradient of atRA ($10^{-5}$ M) was applied to advancing PeA growth cones. Growth cones were found to turn toward the source of atRA (contained within the pipette) with a mean turning angle of $44.0 \pm 4.7 \, ^\circ$ (n=11; Figure 18 A-B). In contrast, the vehicle (control) did not induce growth cone turning toward the pipette ($-6.6 \pm 5.1 \, ^\circ$; n=10; Figure 18 D-E).

Graphical representations of individual growth cone turning values in response to either atRA or the vehicle are shown in Figure 18 C and F respectively. Growth cone turning towards atRA or the vehicle is depicted as a positive turning angle in the graphs whereas growth cone turning away is depicted as a negative turning angle (Figure 18 C, F).
**Figure 18. Growth cones are attracted to a gradient of atRA but fail to turn towards the vehicle.** Representative images depicting the turning response of a PeA growth cone to a local gradient of atRA (A-B) or the vehicle (D-E). The pipette tip is shown on the right side of the image (B; E). Times (t) are given in minutes. Graphs showing the maximum turning angles obtained with atRA (C; n=11) or the vehicle (F; n=10) for each growth cone. Individual growth cones are numbered on the y axis of the graphs. Scale bars = 15μm.
**Growth cones turn towards a gradient of EC23**

After establishing that cultured PeA neurites were attracted to a gradient of atRA (but not to the vehicle), we next sought to identify the response of cultured PeA neurites to various synthetic retinoids in order to gain more insight into the potential role of LymRAR and LymRXR in retinoic acid-mediated growth cone turning. The first synthetic retinoid tested was EC23, which is an analog of atRA with a high affinity for RAR (Charton *et. al.*, 2009). EC23 was tested in order to determine whether it would mimic the chemoattractive effects of retinoic acid. A local gradient of EC23 was applied to advancing growth cones at a concentration of $10^{-5}$ M (in pipette). Growth cones were found to turn toward the source of EC23 with a mean turning angle of $33.7 \pm 6.5 \degree$ (n=10; Figure 19 A-B). A graphical representation of individual growth cone turning angles in response to EC23 is shown in Figure 19 C.

**CH55 induces variable effects on growth cone behaviour**

The second synthetic retinoid tested was CH55, which has high affinity for RAR-$\alpha/\beta$ receptors (Hashimoto *et. al.*, 1990). Variable results on growth cone turning were obtained for CH55 ($10^{-5}$ M) (Figure 20). Representative images of a growth cone showing positive turning towards the focal source of CH55 are shown in Figure 20 A-B whereas Figure 20 C-D show a growth cone turning away from the source of CH55.

A graphical representation of individual growth cone turning angles in response to CH55 is shown in Figure 20E. Although there were variable results for this synthetic retinoid on individual growth cone turning (Figure 20E), CH55 still induced a mean positive turning angle of $15.3 \pm 7.7\degree$ (n=14).
EC23 (Synthetic retinoid and analog of atRA)

A. \( t = 0 \)

B. \( t = 53 \)

C.

Figure 19. Growth cones are attracted to a gradient of the synthetic retinoid, EC23. (A-B) Representative images depicting the turning response of a growth cone to a local gradient of EC23. The pipette tip is shown on the right side of the image (B). C. A graph showing the individual maximum turning angles toward EC23 (n=10). Times (t) are given in minutes. Scale bar = 15μm.
CH55 (Synthetic retinoid that has high affinity for RAR-α/β receptors)

A. t= 0

B. t= 112

C. t= 0

D. t= 30

E.

Figure 20. CH55 induces variable effects on growth cone behaviour. (A-B) Representative images depicting the turning response of a growth cone toward a local gradient of CH55. (C-D) Representative example of a growth cone turning away from CH55. The pipette tip is shown in the upper right hand corner (B; D). E. A graph showing the individual maximum turning angles obtained with CH55 (n=14). Times (t) are given in minutes. Scale bars = 15μm.
Growth cones turn towards a gradient of SR11237, a selective RXR pan-agonist

Previous studies from our lab have shown that an RXR pan-agonist, PA024, was able to produce growth cone turning in both intact and isolated neurites (Carter et. al., 2010). Moreover, the RXR antagonist, HX531, blocked the turning of growth cones towards PA024, as well as to both 9-cis and atRA (Rand, 2012). Yet, the precise role of the RXR in growth cone turning remains unknown and there has been limited study of this nonchordate receptor to date. Unfortunately, the RXR agonist, PA024, is not commercially available and no longer available to us. We therefore sought to determine whether a new commercially available RXR pan agonist, SR11237, would also mimic RA-induced growth cone turning. If so, this would allow us to continue to study the role of RXR in growth cone turning behaviour.

SR11237 was applied to advancing growth cones at either $10^{-5}$ M or $10^{-6}$ M concentrations (similar to those used previously for PA024; Carter et. al., 2010). Growth cones were found to turn toward the source of SR11237 (both concentrations combined) with an overall mean turning angle of $35.5 \pm 6.1^\circ$ (n=10; Figure 21A-B). A graphical representation of individual growth cone turning angles in response to SR11237 at each concentration is shown in Figure 21C. These data indicated that the new, commercially available RXR pan-agonist does indeed mimic the effects of atRA on growth cone behaviour.
SR11237 (Selective RXR pan agonist)

A. \( t = 0 \)

B. \( t = 48 \)

C. Figure 21. Growth cones are attracted to a gradient of the SR11237.

(A-B) Representative images depicting the turning response of a growth cone toward a local gradient of SR11237 \((10^{-5}\text{M})\). The pipette tip is shown in the right hand corner (B). C. A graph showing the individual maximum turning angles toward the SR11237 at two different concentrations \((10^{-5}\text{M} \text{ and } 10^{-6}\text{M}; n=10)\). Growth cones were found to turn toward the source of SR11237 with a mean turning angle of \(31.2 \pm 7.8^\circ\) \((10^{-5}\text{M}; n=5)\) or \(39.8 \pm 9.8^\circ\) \((10^{-6}\text{M}; n=5)\). Times \((t)\) are given in minutes. Scale bars = 15\(\mu\text{m}\).
The mean turning angles in response to each retinoid are summarized in Figure 22. Statistical analysis using a Kruskal-Wallis One Way ANOVA on Ranks showed a significant effect of retinoid treatment ($F_{(4, 50)} = 9.478; p<0.001$). Post-hoc analysis using a Tukey-Kramer test indicated that the overall mean turning angle produced by atRA, EC23, and SR11237 was significantly greater than that produced by the vehicle alone (* $p<0.001$; Figure 22). CH55, a synthetic retinoid that has high affinity for RAR-α/β receptors, did not produce a significantly greater positive turning angle when compared to the vehicle, and was also significantly smaller than that produced by atRA (#$p<0.05$; Figure 22).

Taken together, these data demonstrate that both the synthetic analog of atRA (EC23) and the RXR agonist (SR11237) mimicked the effects of atRA in inducing positive growth cone turning. Though the RAR agonist CH55 could induce growth cone turning of some individual growth cones, overall, the mean growth cone turning angle was not significantly different from the vehicle.
Figure 2. Summary of growth cone responses to retinoids. Summary graph comparing the mean turning angles of growth cones in response to the vehicle, atRA, EC23, CH55, or SR11237. Error bars represent the standard error of the mean (S.E.M.). Statistical analysis used a Kruskal-Wallis One Way ANOVA on Ranks ($F_{(4,50)} = 9.478$ ($p<0.001$) followed by a Tukey-Kramer post-hoc test. The overall mean turning angle produced by atRA, EC23, and SR11237 was significantly larger when compared to that elicited by the vehicle (*$p<0.001$). CH55 did not produce a significantly larger response when compared to the vehicle, and the mean turning angle towards CH55 was significantly smaller than that of atRA (#$p<0.05$). N values for each treatment are provided in brackets.
The Role of Rho GTPase Rac in atRA-induced Growth Cone Turning

It has been previously shown that calcium influx is required for atRA-induced growth cone turning (Farrar et. al., 2009). Rho GTPases are downstream effectors of calcium and are involved in some growth cone responses to other guidance cues (Kaufman et. al., 1998; Rajnicek et. al., 2006). We next investigated the possible role of the Rho GTPase, Rac, in atRA-mediated growth cone turning.

To determine the potential role of Rac, the growth cone turning response to atRA was tested in the presence of the Rac inhibitor, NSC23766 (10^{-4}M final bath concentration), and compared to growth cone responses to atRA in the absence of the Rac inhibitor. In the absence of the Rac inhibitor, the growth cones turn toward the source of atRA with a mean turning angle of 44.0 ± 4.7° (n=11; Figure 23 A-C). However, in the presence of the Rac inhibitor, the growth cones failed to turn toward the source of atRA (mean turning angle: -8.8± 3.7°; n=10; Figure 23 D-F).

In addition to preventing the chemoattractive response to atRA, the Rac inhibitor also appeared to induce a switch in the growth cone response. That is, the growth cones collapsed and retracted during the application of atRA. Growth cone collapse is indicative of an avoidance response to a chemorepulsive guidance cue. However, it is possible that the presence of the Rac inhibitor in the bath may have made the growth cones generally more susceptible to the pressure applied from the pipette and that the growth cone collapse observed was thus not a switch in responsiveness to atRA. In order to rule out this possibility, control experiments were performed whereby the vehicle alone was applied from a pipette to advancing growth cones in the presence of the Rac inhibitor. Though we would not expect the growth cone to exhibit any turning response to the vehicle, if the growth cone collapsing response was merely due to pressure artifact, we would expect to observe this response with
application of vehicle alone. However, the growth cones continued to actively advance in the presence of the vehicle, suggesting that the growth cone collapse in response to atRA was not due to pressure artifact. As expected, the growth cones did not turn toward the vehicle (mean turning angle of 1.2 ± 3.1°; n=10; Figure 23 G-I). Graphs showing the maximum turning angles to atRA in the absence and presence of the Rac inhibitor in the bath, and to the vehicle in the presence of the Rac inhibitor, are shown in Figure 23 C, F, and I, respectively.

A summary of the mean growth cone turning angle are shown in Figure 24. Statistical analysis using a Kruskal-Wallis One Way ANOVA on Ranks showed a significant effect (F (2, 28) = 51.873; p<0.001) and a Tukey-Kramer post-hoc test indicated that the overall mean turning angle toward atRA in the absence of the Rac inhibitor was significantly different from the mean turning angle toward atRA (p<0.001) and the vehicle (p<0.001), in the presence of the Rac inhibitor (Figure 24). These data indicate that Rac may indeed play an important role in mediating atRA-induced growth cone turning.
Figure 23. A switch in the growth cone turning response to atRA in the presence of the Rac inhibitor. Representative images depicting the turning response of a growth cone to atRA in the absence (A-B) or presence (D-E) of the Rac inhibitor in the bath. Note the growth cone collapse shown in E. Vehicle alone was also applied in the presence of the Rac inhibitor (G-H). The pipette tip is shown in the right hand corner (B, E, H). Graphs showing the maximum turning angles of each individual growth cone in each condition are shown in C (n=11), F (n=10), and I (n=10). Individual growth cones are numbered on the y axis of the graphs. The findings shown in C were obtained from Figure 18 and are shown again for comparison. Times (t) are given in minutes. Scale bars = 15μm.
Figure 24. Growth cones fail to turn towards atRA in the presence of Rac inhibitor.

Summary graph comparing the mean turning angles of growth cones in response to atRA in the absence or presence of the Rac inhibitor. Statistical analysis used a Kruskal-Wallis One Way ANOVA on Ranks ($F_{(2, 28)} = 51.873$ ($p<0.001$) followed by with a Tukey-Kramer post-hoc test. Error bars represent the standard error of the mean (S.E.M.). The overall mean turning angle in response to atRA in the absence of the Rac inhibitor was significantly different from the mean turning angle in response to atRA (*$p<0.001$) or the vehicle (*$p<0.001$), in the presence of the Rac inhibitor in the bath. N values for each treatment are provided in brackets.
In order to determine whether the Rac inhibitor did indeed induce a stalling and retraction (collapse) of growth cones in response to atRA, we also determined the extent of growth cone advancement over the duration of each experiment. Graphs show the length of advancement of individual growth cones in response to atRA (in the absence (Figure 25A) or presence (Figure 25 B) of the Rac inhibitor) as well as to the application of the vehicle in the presence of the Rac inhibitor (Figure 25 C). The mean advancement of the growth cones in each condition are summarized in Figure 25 D. Statistical analysis using a Kruskal-Wallis One Way ANOVA on Ranks indicated a statistical difference ($F_{(2,28)}= 11.849$ ($p<0.001$)) and a Tukey-Kramer post-hoc test revealed that in the presence of the Rac inhibitor, growth cone advancement was significantly reduced in response to atRA (-6.7 ± 6.0 μm), compared to in the absence of the inhibitor (25.0 ± 4.1μm; $p<0.001$; Figure 25 D). Furthermore, in the presence of the Rac inhibitor, growth cone advancement in response to atRA was significantly less than to application of the vehicle alone ($p=0.001$; Figure 25 D).

In summary, these findings strongly suggest that the Rac inhibitor not only blocks growth cone turning towards atRA, but also induces a switch in response to atRA, which involved growth cone collapse and retraction (inhibition of growth cone advancement).
Figure 25. Rac inhibitor blocks growth cone advancement in response to atRA application. Graphs showing the length of growth cone advancement in response to atRA in the absence (n=11; A) or presence (n=10; B) of the Rac inhibitor or to the vehicle applied in the presence of the Rac inhibitor (n=10; C) over a time course of 50 to 60 minutes. Individual growth cones are numbered on the x axis of the graphs. D. Summary graph comparing the mean length of advancement of neurites in each condition. Error bars represent the standard error of the mean (S.E.M.). Statistical analysis was performed using a Kruskal-Wallis One Way ANOVA on Ranks ($F_{(2, 28)} = 11.849$; *p<0.001) followed by a Tukey-Kramer post-hoc test. In the presence of the Rac inhibitor, growth cone advancement was significantly reduced in response to atRA (#p<0.001 compared to in the absence of Rac inhibitor; *p=0.001 compared to vehicle). N values for each treatment are provided in brackets.
The Role of Rac in EC23-induced Growth Cone Turning

We have previously determined that EC23 mimics atRA-induced growth cone turning, and the data suggests that Rac may be involved in growth cone turning towards atRA. Hence, my next aim was to determine whether the Rac inhibitor would also abolish EC23-induced growth cone turning.

EC23 normally induces growth cone turning with a mean angle of 33.7 ± 6.5μm (Figure 26 A-B; n=10). In the presence of the Rac inhibitor, the growth cones continued to turn towards the focally applied EC23, albeit it with a smaller mean turning angle of 17.9 ± 6.4° (Figure 26 D-E; n=10). Furthermore, growth cones continued to advance following application of EC23, and did not exhibit growth cone collapse as they had previously in response to atRA. Graphs showing the maximum turning angles of each individual growth cone in response to EC23 in the absence and presence of the Rac inhibitor are shown in Figure 26 C and F respectively.
**EC23 applied in the absence of Rac inhibitor**

A. t= 0  

B. t= 53  

C.  

**EC23 applied in the presence of Rac inhibitor**

D. t= 0  

E. t= 40  

F.  

**Figure 26. Rac inhibitor does not block EC23 mediated growth cone turning.** Representative images depicting the turning response of a growth cone to a local gradient of the EC23 in the absence (A-B) or presence (D-E) of the Rac inhibitor. The pipette tip is shown on the right side of image (B, E). Graphs showing the maximum turning angles of individual growth cones toward EC23 in the absence (C; n=10) or presence (F; n=10) of the Rac inhibitor. Individual growth cones are numbered on the y axis of the graphs. The findings shown in C were obtained from Figure 19 and are shown again for comparison. Times (t) are given in minutes. Scale bars = 15μm.
In order to compare the effects of the Rac inhibitor on the chemoattractive responses of both atRA and EC23, the growth cone turning angles are summarized in Figure 27. Statistical analysis was performed using a Two Way ANOVA and revealed a significant interaction effect between the bath treatment (Rac inhibitor) and the retinoid applied to the growth cone (F_{1,37} = 11.69; p =0.002). As shown previously, a Holm–Bonferroni Sequential Correction post-hoc test showed that there was a significant reduction in growth cone turning towards atRA in the presence of the Rac inhibitor as compared to in its absence (p<0.0125, Holm-Bonferroni sequential corrected p value; Figure 27). Furthermore, in the presence of the Rac inhibitor, the growth cone turning angle was significantly smaller in response to atRA than in response to EC23 (p<0.017, Holm-Bonferroni sequential corrected p value; Figure 27). Although the mean turning angle in response to EC23 was smaller in the presence of the Rac inhibitor, this reduction did not reach statistical significance (p>0.025, Holm-Bonferroni sequential corrected p value; Figure 27).
Figure 27. Rac inhibitor blocks growth cone turning in response to atRA but not in response to EC23. Summary graph comparing the mean turning angles of growth cones in response to atRA or EC23, in the presence or absence of the Rac inhibitor. Error bars represent the standard error of the mean (S.E.M.). Statistical analysis used a Two Way ANOVA and revealed a significant interaction effect between the bath treatment (Rac inhibitor) and the retinoid applied ($F_{(1,37)} = 11.69; p = 0.002$). A Holm–Bonferroni Sequential Correction post-hoc test indicates a significant reduction in the mean growth cone turning angle in response to atRA in the presence of the Rac inhibitor as compared to in its absence (*$p<0.0125$). There was also a significant reduction in the mean growth cone turning angle in response to atRA compared to EC23 in the presence of the Rac inhibitor (#$p<0.017$). N values for each treatment are provided in brackets.
In order to determine whether the Rac inhibitor induced any stalling and retraction of growth cones in response to EC23, we also determined the extent of growth cone advancement over the duration of each experiment. Graphs showing the length of advancement of individual growth cones in response to EC23 in the absence and presence of the Rac inhibitor are shown in Figure 28 A and B, respectively. Furthermore, a summary graph comparing the growth cone advancement in response to EC23 compared with atRA are also summarized in Figure 28 C. A Two Way ANOVA revealed a significant interaction effect of bath treatment (Rac inhibitor) and the retinoid applied (F (1, 37) = 12.06; p =0.001). A Holm–Bonferroni Sequential Correction post-hoc test confirmed a significant reduction in growth cone advancement in response to atRA in the presence of the Rac inhibitor, compared to in its absence (*p<0.0125, Holm-Bonferroni sequential corrected p value; Figure 28); whereas there was no such reduction for EC23 (p=0.792 (>0.025, Holm-Bonferroni sequential corrected p value); Figure 28). However, there was a significant reduction in growth cone advancement in response to atRA compared to advancement in EC23 in the presence of the Rac inhibitor (#p<0.017, Holm-Bonferroni sequential corrected p value; Figure 28).

In summary, these data suggest that the Rac inhibitor induces a significant inhibition of growth cone turning responses to atRA, but not to the synthetic retinoid EC23. Moreover, the data have shown that the Rac inhibitor induces a switch in the growth cone response to atRA, from chemoattraction to growth cone collapse and retraction. This inhibition of growth cone turning, or switching of growth cone response to chemorepulsion did not occur in response to the synthetic retinoid EC23 in the presence of the Rac inhibitor.
Figure 28. Rac inhibitor blocks growth cone advancement in response to atRA but not in response to EC23. Graphs showing the length of growth cone advancement in response to EC23 in the absence (n=10; A) or presence (n=10; B) of the Rac inhibitor. Individual growth cones are numbered on the x axis of the graphs. C. Summary graph comparing the mean length of advancement of neurites in response to atRA or EC23, in the presence or absence of the Rac inhibitor. Error bars represent the standard error of the mean (S.E.M.). A Two Way ANOVA revealed a significant interaction effect between the bath treatment (Rac inhibitor) and the retinoid applied (F (1, 37) = 12.06; p =0.001). A Holm–Bonferroni Sequential Correction post-hoc test indicates the Rac inhibitor significantly reduced growth cone advancement in response to atRA (*p<0.0125). There was also a significant reduction in growth cone advancement in response to atRA compared with EC23 in the presence of the Rac inhibitor (#p<0.017). N values for each treatment are provided in brackets.
The Rac Inhibitor Does Not Inhibit atRA-Induced Growth Cone Turning of Isolated Growth Cones

It has been shown that atRA-induced growth cone turning is transcriptionally independent and relies on the local synthesis of proteins in growth cones (Farrar et al., 2009). That is, previous studies have shown that growth cones isolated from the cell body continue to respond to atRA (Farrar et al., 2009). An advantage of using cultured Lymania neurons is that neurites can survive transection from the cell body and can continue to grow for many hours. After identifying that the Rac inhibitor induced a switch in the response of growth cones in response to atRA, my next aim was to determine whether the inhibition of Rac would also attenuate growth cone turning and advancement of transected growth cones. This could shed light on whether the involvement of Rac in growth cone turning relies on communication from the cell body.

The response of transected growth cones to atRA was first tested, to ensure repeatability of previous findings (Farrar et al., 2009). A local gradient of atRA ($10^{-5}$ M) was applied to advancing transected growth cones (in the absence of the Rac inhibitor). Growth cones were found to turn and advance toward the source of atRA (pipette) with a mean turning angle of $39.1 \pm 5.4^\circ$ (n=13; Figure 29 A-B). Interestingly, in the presence of the Rac inhibitor, the transected growth cones continued to turn and advance towards the focally applied atRA with a mean turning angle of $25.7 \pm 4.5^\circ$ (n=31; Figure 29 D-E). Graphs showing maximum turning angles of each individual transected growth cone in response to atRA, in the absence or presence of the Rac inhibitor, are shown in Figure 29 C and F respectively.
Transected growth cone response to atRA in the absence of Rac inhibitor

A. t=0

B. t=30

C.

Transected growth cone response to atRA in the presence of Rac inhibitor

D. t=0

E. t=35

F.

Figure 29. Rac inhibitor does not block transected growth cone turning towards atRA.
Representative images depicting the turning response of a transected growth cone to a local gradient of atRA, in the absence (A-B) or presence of the Rac inhibitor in the bath (D-E). The pipette tip is shown on the right side of the images (B, E). Graphs showing the maximum turning angles of individual growth cones to atRA, in the absence (C; n=13) or presence (F; n=12) of the Rac inhibitor in the bath. Individual growth cones are numbered on the y axis of the graphs. Times (t) are given in minutes. Scale bars = 15μm
The mean turning angles of intact or transected growth cones towards atRA, in the absence or presence of the Rac inhibitor, are summarized in Figure 30. A Two Way ANOVA revealed a significant interaction effect between the bath treatment (Rac inhibitor) and the condition of the growth cone ($F_{(1,42)} = 17.032; \ p < 0.001$). Holm–Bonferroni Sequential Correction post-hoc test confirmed a significant reduction in intact growth cone turning towards atRA in the presence of the Rac inhibitor as compared to in its absence ($p < 0.0125$, Holm-Bonferroni sequential corrected p value; Figure 30). The growth cone turning of intact neurites to atRA in the Rac inhibitor was significantly reduced compared to that of transected growth cones in the same condition ($p < 0.0125$, Holm-Bonferroni sequential corrected p value; Figure 30).
Figure 30. Rac inhibitor does not block transected growth cone turning towards atRA.
Summary graph comparing the mean turning angles of intact and transected growth cones in response to atRA, in presence or absence of the Rac inhibitor. Error bars represent the standard error of the mean (S.E.M.). A Two Way ANOVA revealed a significant interaction effect between the bath treatment with the Rac inhibitor and the transection of the growth cone ($F_{(1,42)} = 17.032; p < 0.001$). A Holm–Bonferroni Sequential Correction post-hoc test indicates a significant reduction in intact growth cone turning towards atRA in the presence of the Rac inhibitor as compared to in its absence (*p<0.0125). There was also a significant difference between intact and transected growth cone turning in response to atRA (*p<0.0125). N values for each treatment are provided in brackets.
In order to determine whether the Rac inhibitor induced a stalling and retraction of transected growth cones in response to atRA, we also determined the extent of growth cone advancement over the duration of each experiment. Graphs showing the length of advancement of individual growth cones to atRA in the absence and presence of the Rac inhibitor are shown in Figure 31 A and B, respectively. Furthermore, a summary graph comparing the advancement of intact, compared with transected growth cones, is shown in Figure 31 C. A Two Way ANOVA revealed a significant interaction effect of bath treatment (Rac inhibitor) and the condition of the growth cone (intact or transected) \((F_{(1, 42)} = 14.478; p <0.001\)). A Holm–Bonferroni Sequential Correction *post-hoc* test revealed a significant reduction in intact growth cone advancement in response atRA in the presence of the Rac inhibitor as compared to in its absence (p<0.0125, Holm-Bonferroni sequential corrected p value; Figure 31). There was also a significant reduction in the advancement of intact growth cones towards atRA compared to transected growth cones, in the presence of the Rac inhibitor (p<0.017, Holm-Bonferroni sequential corrected p value; Figure 31).

In summary, studies have shown that the Rac inhibitor induces a switch in growth cone response (of intact growth cones) to atRA from chemoattraction to chemorepulsion. That is, growth cones collapsed and retracted in response to atRA when the Rac inhibitor was present. Interestingly, however, the Rac inhibitor did not induce the same significant effects on the response of growth cones to the synthetic retinoid EC23. Moreover, the Rac inhibitor did not induce the same effects on growth cones transected from the cell body. These data suggest that the involvement of Rac in growth cone turning may differ depending on whether the turning is induced by endogenous or synthetic retinoids, and also whether the growth cones have maintained communication with the cell body.
Transected growth cone response to atRA:

A. Absence of Rac inhibitor

B. Presence of Rac inhibitor

<table>
<thead>
<tr>
<th>Pipette:</th>
<th>atRA</th>
<th>atRA</th>
<th>atRA</th>
<th>atRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bath:</td>
<td>---------</td>
<td>NSC23766</td>
<td>---------</td>
<td>NSC23766</td>
</tr>
<tr>
<td>Neurite condition:</td>
<td>Intact</td>
<td>Intact</td>
<td>Transected</td>
<td>Transected</td>
</tr>
</tbody>
</table>

C. Summary graph comparing the mean length of advancement of intact or transected growth cones in response to atRA, in the presence or absence of the Rac inhibitor. Error bars represent the standard error of the mean (S.E.M.). A Two Way ANOVA revealed a significant interaction effect between the bath treatment with the Rac inhibitor and the condition of the growth cone (intact or transected) (F(1,42) = 11.478; p < 0.001). A Holm–Bonferroni Sequential Correction post-hoc test indicates that the Rac inhibitor significantly reduced intact growth cone advancement to atRA (*p<0.0125). There was also a significant reduction in intact growth cone advancement towards atRA compared with transected growth cones (#p<0.017) in the presence of the Rac inhibitor. N values for each treatment are provided in brackets.

Figure 31. Rac inhibitor blocks growth cone advancement of intact, but not transected, neurites in response to atRA. Graphs showing the length of transected growth cone advancement in response to atRA in the absence (n=13; A) or presence (n=12; B) of the Rac inhibitor. Individual growth cones are numbered on the x axis of the graphs. C. Summary graph comparing the mean length of advancement of intact or transected growth cones in response to atRA, in the presence or absence of the Rac inhibitor. Error bars represent the standard error of the mean (S.E.M.).
3.04 Discussion

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

Retinoic acid is a molecule that has been implicated in several developmental and regenerative processes, including acting as a chemoattractant to guide regenerating axons in vertebrates (Dmetrichuk *et. al.*, 2005) and invertebrates such as *Lymnaea* (Dmetrichuk *et. al.*, 2006; 2008). The main effects of retinoic acid in regeneration are thought to occur via signalling through receptors, which include RARs and RXRs. Recently, in the mollusc *Lymnaea*, an RXR and a putative RAR have been cloned (Carter *et. al.*, 2010, 2015). However, there has been limited study of these receptors to date, and the mechanisms underlying the tropic effects of retinoic acid in *Lymnaea* remain largely unknown. Since identifying the localization of *Lym*RAR and *Lym*RXR in the neurites and specifically in the growth cones of cultured neurons (Carter *et. al.*, 2010; 2015), a role for both retinoid receptors in retinoic acid-mediated growth cone guidance has been proposed. Hence, we sought to further investigate the response of cultured *Lymnaea* PeA neurites to various synthetic retinoids, in order to gain more insight into the potential role of *Lym*RAR and *Lym*RXR in retinoic acid-mediated growth cone turning.

Carter *et. al.* (2010) first tested a RXR agonist, PA024, and found that it was able to mimic retinoic acid and induce growth cone turning in both intact and isolated neurites of *Lymnaea*. However, this agonist is no longer available for use. Furthermore, no RAR agonists have previously been shown to mimic the action of atRA. Therefore, we chose to test the effects of the RAR agonists: EC23 and CH55, as well as a new commercially available RXR pan agonist, SR11237, on the growth cone turning behaviour of *Lymnaea* neurites.
We determined that both RAR and RXR agonists can indeed mimic the chemoattractive effects of atRA in cultured regenerating _Lymnaea_ neurons. We showed that _Lymnaea_ PeA motoneurons are attracted to EC23, an analog of atRA, and SR11237, an RXR pan-agonist. Indeed, EC23 has been shown in previous vertebrate studies to display similar biological activity to atRA (Christie _et. al._, 2008; Maltman _et. al._, 2009), as has SR11237 to 9-cis retinoic acid (Lehmann _et. al._, 1992; Zhou _et. al._, 2010). However, the synthetic retinoid, CH55 produced ambiguous results in the growth cone turning assays. Some growth cones turned towards CH55, while others did not. Although the mean turning angle towards CH55 was positive, it was not significantly different from the growth cone response to the vehicle alone. This ambiguity contradicts previous findings in vertebrates that found CH55 exhibited potent retinoid-like activity in both stimulatory and inhibitory biological processes (Sato, 1988). For example, CH55 induced differentiation of embryonal carcinoma F9 cells and inhibited the growth of melanoma S91 cells, in a similar manner to atRA (Jetten _et. al._, 1987).

Differences in the binding affinity of EC23 and CH55 to _LymRAR_ may account for the difference in the responses observed. Although both these RAR agonists show higher binding affinities to RAR compared to atRA in vertebrates (Gambone _et. al._, 2002; Hashimoto _et. al._, 1990), the synthetic retinoids may not bind as efficiently with _LymRAR_ and CH55 may bind less efficiently than EC23. Indeed, previous studies in _Lymnaea_ have also found EC23 to be more potent than CH55 in disrupting embryonic development (de Hoog, 2015). These findings suggest that CH55 may have poor binding affinity to retinoid receptors in _Lymnaea_, which may account for the lack of an overall significant positive effect on growth cone turning (despite some individual positive growth cone responses).
It is also possible that the different synthetic retinoids might activate different intracellular signalling pathways downstream of the retinoid receptors, compared to atRA, and this might also account for differences in the turning responses observed. Although synthetic retinoids can elicit the same biological response as retinoic acid, they might do so through different/diverging cellular mechanism(s). For example, this has been suggested previously for CH55, which was found to activate a different intracellular signalling pathway than atRA in rabbit tracheal epithelial cells (Jetten et. al., 1987). Moreover, previous findings have suggested that retinoic acid may also induce signal transduction pathways independent of binding to RAR and RXR. For example, retinoic acid can activate cyclic adenosine monophosphate response element-binding protein (CREB) independent of RAR and RXR in human tracheobronchial epithelial cells (Aggarwal et. al., 2006) and direct interactions between retinoic acid and protein kinase A (PKA) have been documented (Wong et. al., 2006; Saito et. al., 2010). Therefore, in addition to binding to RAR and/or RXR, atRA may also be exerting additional or parallel actions via receptor-independent pathways. Taken together, these findings still support a potential role for LymRAR and LymRXR in retinoic acid-mediated growth cone turning, though further studies will be needed to examine the pathways involved in more detail.

The role of Rac in atRA-induced growth cone turning

Although previous studies have provided some evidence for the role of neuritically localized retinoid receptors in the chemoattractive effects of retinoic acid, far less is known of potential downstream effectors in this response. To date, retinoic acid-mediated growth cone turning has been shown to require both local protein synthesis and calcium influx (Farrar et. al., 2009). However, the signalling cascades which might link calcium influx to
the regulation of cytoskeletal dynamics involved in growth cone turning are not currently known. Rho GTPases are known downstream effectors of calcium and are involved in growth cone responses to other guidance cues (Kaufman et. al., 1998; Rajnicek et. al., 2006). However, whether Rho GTPases play a role in retinoic acid-induced chemoattraction had not previously been studied. My aim was to determine the possible role of the Rho GTPase Rac in retinoic acid-mediated growth cone turning in regenerating *Lymnaea* neurons.

Previous studies have shown that growth cones of *Lymnaea* PeA neurites turn and extend towards focally applied atRA (Farrar et. al., 2009). However, in the presence of the Rac inhibitor, we showed an inhibition of growth cone turning to atRA. This demonstration, that Rac prevented chemotropic growth cone attraction, is in agreement with several studies that have also suggested a positive role for Rac in the directional migration of growth cones. For example, genetic disruption of Rac activity was found to result in growth cone pathfinding defects of motor axons in *Drosophila* embryos (Kaufman et. al., 1998) and of spinal neurons in *Xenopus* embryos (Rajnicek et. al., 2006). Rac has also been found to mediate growth cone attraction to netrin in rat embryonic spinal cord explants (Li et. al., 2002).

In addition to the inhibition of growth cone turning, a switching in the response to atRA was observed in the presence of the Rac inhibitor. The growth cones were found to collapse and retract in the presence of atRA, which is indicative of chemorepulsion. This chemorepulsive response was specific to application of atRA, as it did not occur on application of the vehicle, and thus was not a pressure artifact. Similarly, previous studies have also shown a switch in the response of *Xenopus* growth cones to the chemoattractant BDNF, following the inhibition of all three Rho GTPases, Rac, Cdc42, and Rho (Yuan et. al., 2003). Other studies have also shown a similar switch in *Xenopus* growth cones’
response after pharmacological manipulation of cyclic adenosine monophosphate (cAMP) or cAMP-dependent kinases, such as PKA (Song et al., 1998). That is, in the presence of a competitive analogue of cAMP or a specific inhibitor of PKA, a gradient of BDNF (or acetylcholine), which normally triggered an attractive turning response, induced a repulsive turning response of *Xenopus* spinal growth cones (Song et al., 1997). As there is evidence that Rac activation can be controlled by cAMP/PKA mediated-signalling (in the directed migration of carcinoma cells; O’Connor and Mercurio, 2001), it is possible that the switch in response to atRA observed here may result from an interaction between Rac and cAMP levels within the growth cone.

Furthermore, recent studies postulated that attractive growth cone turning entails the accumulation of functional membrane receptors and cytoskeletal components on the side of the growth cone closest to the chemoattractant (Bouzigues *et. al.*, 2007). Although, in *Lymnaea*, retinoid receptors were detected in the membrane and cytoplasmic fractions of neurons (Carter *et. al.*, 2010; 2015), whether retinoid receptors are redistributed to the side of the growth cone closest to retinoic acid, and where (cytoplasm or membrane) retinoic acid binds retinoid receptors to initiate growth cone turning, need to be determined. If retinoid receptors are indeed asymmetrically redistributed during attractive growth cone turning, Rho GTPases may be involved in facilitating their transportation, through cytoskeletal-dependant vesicle transport. Several proteins that regulate the assembly of the actin cytoskeleton, including actin-binding proteins, have been shown to be downstream effectors of Rho GTPases (Hall, 2005). Nonetheless, in summary, these findings suggest that intracellular signal transduction pathways involving Rac, may mediate the response of growth cones to guidance cues such as retinoic acid.
The role of Rac in EC23-induced growth cone turning

My data suggests that Rac may be a downstream effector of atRA to induce growth cone turning. Since EC23 was shown to mimic atRA-induced growth cone turning, we also sought to determine whether the Rac inhibitor may also be involved in EC23-mediated growth cone turning. However, in the presence of the Rac inhibitor, the growth cones continued to advance in response to EC23 and did not exhibit growth cone collapse and retraction as they had in response to atRA. Although there was substantial reduction in the mean turning angle towards EC23 in the presence of the Rac inhibitor (as compared to in its absence), the growth cones continued to turn towards EC23.

It is possible that the endogenous ligand, atRA, may be acting through intracellular signalling pathways different from that of EC23, which may explain the differences in the growth cone responses observed here. As mentioned previously, the synthetic retinoid CH55 was shown to activate different intracellular pathways than atRA (Jetten et. al., 1987). This may also be true for EC23, which may activate intracellular pathways that are independent of Rac. Moreover, the effects mediated by atRA may not be exclusively through the RAR or RXR receptor, and these non-receptor mediated effects may require Rac signalling. For example, there is evidence for the direct modulation of protein kinase C (PKC) signalling by retinoic acid, and PKC has a retinoic acid-binding site (Radomsinska-Pandya et al., 2000; Ochoa et al., 2003). Retinoic acid can also activate cAMP response element-binding protein (CREB) independent of RAR and RXR in human tracheobronchial epithelial cells (Aggarwal et al. 2006). Interestingly, both PKC and CREB have previously been found to interact with Rho GTPases (Slater et. al., 2001; Sordella et. al., 2002; Kanazawa et. al., 2014).

Alternatively, EC23 may be having more potent or prolonged effects on downstream activators that the Rac inhibitor could not overcome. For example, EC23 has been found to
be more potent than atRA in inducing neural differentiation in human pluripotent embryonic stem cells (Christie et al., 2008). EC23 induces higher CRABP-I expression (a protein that sequesters retinoic acid), compared to atRA (Maltman et al., 2009). Although there is currently no evidence that EC23 could also be sequestered by CRABP-I, the differential effects of EC23 on CRABP-I expression, compared to atRA, may explain enhanced activity associated with EC23. EC23 may also exhibit an increased resistance to cellular metabolism compared to atRA. For example, there appears to be no evidence that EC23 is degraded by CYP26A1, an enzyme that is responsible for degrading endogenous retinoids (Reijntjes et al., 2005), and EC23 elicits a weaker induction of CYP26A1 expression, compared to atRA (Haffez et al., 2007). Therefore, these differences in binding affinities or metabolism might result in prolonged effects of EC23, compared to atRA, and may help explain the persistence of the growth cone turning in response to EC23, even after Rac inhibition.

The role of Rac in atRA-induced turning of isolated growth cones

Previous studies have shown that Lymnaea growth cones, isolated from the cell body, continue to exhibit turning in response to atRA (Farrar et al., 2009). These growth cone responses to atRA were shown to rely on calcium influx and the local synthesis of proteins (Farrar et al., 2009). After identifying that the Rac inhibitor induced a switch in the response of growth cones towards atRA, we also determined whether the inhibition of Rac would induce similar effects on transected growth cones. Unlike the intact growth cones, the transected growth cones continued to turn and advance towards the focally applied atRA in the presence of the Rac inhibitor. However, a substantial (albeit non-significant) reduction in the mean turning angle was observed in the Rac inhibitor, which suggests that Rac might still
play some role in the transected growth cones, but not to the same extent as it does in intact growth cones.

It is possible that transection of the growth cones might have initiated a compensatory mechanism that may be either independent of Rac, or that relies on alternative guanine nucleotide exchange factors (GEFs) to activate Rac. Rac is activated when GEFs catalyze the exchange of GDP for GTP (Gao et al., 2004). The Rac inhibitor used in my experiments, NSC23766, supposedly binds to Rac and targets the interaction between Rac and a subset of its GEFs: Tiam1 and Trio. However, there are several other GEFs that have been shown to activate Rac such as phosphatidylinositol (3,4,5)-trisphosphate-dependent Rac exchanger 2 (P-Rex2) (Donald et al., 2004). It could be possible that alternative GEFs may have allowed the activity of Rac to persist. It could also be possible that transection of the growth cones could have impeded the effects of the Rac inhibitor. That is, inhibiting Rac activity in the cell might be facilitated by cross talk with upstream effectors or genomic processes. Previous studies have confirmed communication between the growth cones and the cell body through rapid generation of calcium waves (Guan et al., 2007) and active transport of vesicles to and from the cell body (Denburg et al., 2005). These vesicles could be transporting regulatory proteins and signalling ligands that may have been facilitating inhibition of Rac by the NSC23766.

In summary, these studies demonstrated the ability of a number of synthetic retinoid agonists to mimic atRA to induce growth cone turning. We also showed for the first time that the intracellular signalling pathway involved in growth cone turning is likely to involve the Rho GTPase, Rac. However, the involvement of Rac in growth cone turning may differ depending on whether the turning is induced by endogenous or synthetic retinoids, and also whether the growth cones have communication with the cell body.
Chapter 4:
Conclusions and Perspectives
Understanding the processes underlying neuronal regeneration is a major goal of research. Many signaling molecules that were once thought to only be important during neural development are now known to be just as important postnatally, including during neural regeneration. Due to the preservation of these signalling molecules in development and regeneration, many believe that neural regeneration may involve re-activation of the developmental signaling pathways. Therefore, research on neuronal regeneration may not only be used to understand the signalling pathways involved in enabling regeneration, but also possibly the signalling pathways involved in development.

Retinoic acid is one of the signalling molecules that has been found to have a conserved role in neural development and regeneration. Retinoic acid signalling has been found to be involved in neuronal patterning, neural differentiation and axon outgrowth in the developing nervous system (Maden, 2007). Retinoic acid synthesis and signalling has also been shown to be up-regulated following injury in several species (Viviano et al. 1995; Zhelyaznik et al., 2003). Following nerve damage, this molecule has the capacity to induce neural regeneration (Dmetrichuk et al., 2005; Dmetrichuk et al., 2006) as well as guide axonal processes (Dmetrichuk et al., 2006; Dmetrichuk et al., 2008). Moreover, the downregulation of retinoic acid signalling has been suggested to be at least partly involved in the loss of capacity to regenerate CNS neurons in some adult vertebrates. For example, supplementation with retinoic acid was not sufficient to induce neurite outgrowth in the adult mouse spinal cord, yet, neurite outgrowth was accomplished following the introduction of the retinoic acid receptor gene, RARβ2 (Corcoran et al., 2002). Although retinoic acid and its various signaling components have been identified in adult species, and its role in adult regeneration has been implicated, the exact processes involved have not been fully
determined. Therefore, the fundamental pathway(s) through which retinoic acid elicits its effects during regeneration still require further characterization.

Adult invertebrates that retain the ability to regenerate their CNS have been useful model systems to study regeneration. *Lymnaea stagnalis*, in particular, is well-established as a model system for regeneration. Unlike the adult mammalian CNS, the CNS of adult *Lymnaea stagnalis* is capable of extensive regeneration. Moreover, single identified neurons that have a well characterized morphology and function in the CNS have been successfully cultured from this model system. This model system can, therefore, be used to investigate the signalling pathways through which retinoic acid elicits its effects during neural regeneration.

The CNS of adult *Lymnaea stagnalis* has been used in our lab to investigate the effect of retinoic acid on neurite outgrowth during regeneration. J. Simmons had determined that *Lymnaea* CNS floating on the surface of DM could exhibit neurite outgrowth (*unpublished findings*). When testing the effects of retinoid isomers on the neurite outgrowth from the floating CNS, my results were not consistent across concentrations. Despite this, the data in this thesis still suggest that 9-cis RA, unlike atRA, might be able to enhance neurite outgrowth from the floating CNS. There are several advantages to using this intact CNS preparation in studying the effects of retinoic acid on neurite outgrowth. One advantage is that studying the intact CNS allows us to study multiple cell types and axons within nerve bundles simultaneously, rather than just individual sensory or motor neurons (as with cell culture). Another advantage of using this preparation is the ability to observe large-scale changes (unlike with cultured neurons) which would more closely mimic an *in vivo* response. Promising findings from our lab (*unpublished observations*) have also demonstrated that outgrowth from the floating CNS could be harvested on coverslips for future immunostaining studies. Hence, there are several advantages to continue using the floating CNS preparation.
to investigate the effects of retinoic acid on neurite outgrowth from the *Lymnaea* CNS. The experimental procedures for this novel preparation can be refined in future experiments to obtain more consistent results and verify whether there is indeed a trophic effect of 9-*cis* RA. In future experiments, it might be best to change the concentration of retinoic acid, or to add retinoic acid daily throughout the duration of the experiment to avoid degradation. Alternatively, various synthetic retinoids that mimic endogenous retinoids but are degraded less quickly could be used (Haffez *et. al.*, 2017).

The floating CNS model may also be useful for looking at the directional behaviour of neurite outgrowth emanating from cut nerves towards floating targets, such as neighboring nerve fragments and/or ganglia. In previous research, it was suggested that growth cone migration and axon elongation relies on adhesive contacts with solid substrates to occur (Gomez and Letourneau, 2014). However, we clearly showed here, for the first time, that growth cones can still sense atRA and respond in the absence of substrate adhesion. The tropic effects of retinoic acid were also only previously shown on *Lymnaea* motorneurons (Pedal A and Visceral F) in culture. However, the data in this thesis suggested that other cell types (though not identifiable) might also be responsive to atRA. Nevertheless, the floating CNS of *Lymnaea* may not be considered an optimal model system to investigate the directional behaviour of single growth cones in response to guidance cues. Growth cones of the CNS were found to be slow and sensitive to external disruptions.

Cultured *Lymnaea* neurons were found to be a better model system for investigating the cellular mechanisms underlying the tropic effects of retinoic acid on neurite outgrowth. Large growth cones could be observed at the tips of regenerating neurites of cultured cells. This makes it possible to work directly on these growth cones and to easily manipulate and observe changes in their morphology and behaviour. Moreover, the growth cones of cultured
Lymnaea motorneurons can continue to grow after transection, allowing us to study the autonomy of the growth cone (Farrar et. al., 2009). Another advantage of using this model system is the ability to observe local effects of guidance cues and the involvement of protein synthesis, as previously done by Farrar et. al. (2009). Cultured neurons may, therefore, be the optimal system to continue to investigate the fundamental pathway(s) through which retinoic acid elicits its tropic effects.

Previous findings from cultured Lymnaea neurons have shown that both retinoic acid isomers similarly induce and enhance neurite outgrowth as well as induce growth cone turning in Lymnaea neurons, in vitro (Dmetrichuk et. al., 2006; 2008). Previous findings have strongly suggested an important role for both retinoid receptors, LymRXR and LymRAR, in the chemoattractive effects of retinoic acid on growth cone guidance (Carter et. al., 2010, 2015). In this thesis, we have provided further evidence suggesting that the RXR and RAR may indeed be involved in this chemoattractive response to retinoic acid, as various synthetic retinoid receptor agonists mimicked effects of atRA. However, binding studies still need to be performed to verify the binding of the synthetic retinoids to the various retinoid receptors in Lymnaea.

Aside from the proposed role of retinoid receptors, far less is known of potential downstream effectors involved in the chemoattractive effects of retinoic acid. Retinoic acid-mediated growth cone turning has been shown to require both local protein synthesis and calcium influx (Farrar et. al., 2009). However, the downstream effectors that are involved in growth cone turning have not been identified. We have shown, for the first time in any species, that the intracellular signaling pathway involved in retinoic acid-induced growth cone turning involves the Rho GTPase, Rac. My findings, however, have shown that the involvement of Rac in growth cone turning may differ depending on whether the turning is
induced by endogenous or synthetic retinoids, and whether the growth cones are still attached to the cell body.

The mechanism of action of Rac and the signaling pathway involved will still need further study and identification. We found that Rac inhibition induces a switch in growth cone response to atRA (from chemoattraction to chemorepulsion). A similar switch in the Xenopus’s growth cone response to the chemoattractant BDNF was observed after the inhibition of all three Rho GTPases (Yuan et. al., 2003), or after pharmacological manipulation of cAMP or cAMP-dependent kinases (Song et. al., 1998). These findings suggested that the switch in response to atRA may be a result of an interaction between Rac and cAMP levels within the growth cone. Therefore, future studies could investigate a possible interaction between Rac and cAMP, by inhibiting intracellular cAMP production during growth cone turning behaviors before and after Rac inhibition. Additionally, future studies will need to examine possible downstream effectors through which Rac likely regulates cytoskeletal changes in the growth cone. Future experiments could also investigate the potential role of other Rho GTPases such as Cdc42 or Rho (which has been suggested to mediate growth cone collapse) in retinoic acid-induced growth cone turning behaviour in Lymnaea. Furthermore, retinoid isomers (atRA and 9-cis RA) have different effects on the intracellular calcium levels (Vesprini et. al., 2015), and may activate different intracellular signaling pathways, in Lymnaea neurons. Therefore, potential differences in the role of Rho GTPases in growth cone turning behaviour to different isomers could also be investigated.

Individual growth cone turning assays with retinoic acid have only previously been reported with invertebrate neurons. However, there is now evidence that atRA and 9-cis RA have similar trophic and chemotropic effects on cultured embryonic spinal cord neurons of Xenopus laevis (Rand et. al., 2017). Rand et. al. (2017) also provided evidence suggesting a
potential role for the RARβ sub-type and an RXR in the atRA and 9-cis RA-induced growth cone turning, respectively. *Lymnaea* neurons, however, were still easier to work with to investigate the tropic effects of retinoic acid on neurite outgrowth than cultured *Xenopus* neurons. Taken together, these findings support a role for retinoid signalling in guiding neurite outgrowth during regeneration, and could suggest a conservation of retinoid signalling pathways between vertebrates and invertebrates.

Currently, we are among the few researchers (to our knowledge) investigating the cellular mechanisms involved in the chemoattractive effects of retinoic acid on growth cone guidance. The novel findings presented in this thesis provide a basis for future studies to continue to investigate the intracellular signalling pathways involved in the effects of retinoic acid on neurite outgrowth. The research conducted in our lab, combined with published findings, can help discern these mechanisms and ultimately provide progress in understanding processes involved in the effects of retinoic acid during regeneration.
Appendix 1:

**Fewer nerves exhibit neurite outgrowth at lower concentrations of atRA.** The proportion (%) of floating nerves that exhibited outgrowth in the presence of DMSO, 9-*cis* RA, and atRA in the bath was determined at both concentrations (10⁻⁶M and 5x10⁻⁷M). Only floating nerves were counted, as those submerged do not exhibit outgrowth.

These data were re-analyzed, with the kind help of G. Tattersall, using generalized linear mixed effect models, accounting for the influence of brain “identity” (i.e. which CNS it originated from) as a random effect. Statistical analyses were performed using R software (© The R Foundation). When nerve identity was accounted for, a type-II Wald Chi-square test showed a significant effect of condition (retinoid treatment) but only when the concentration of retinoid was considered. The findings of this R analysis were similar to those mentioned previously (on page 45), which showed there was a significant effect of the condition (retinoid treatment) but only at lower concentrations.

<table>
<thead>
<tr>
<th></th>
<th>Chi square value</th>
<th>Degrees of Freedom</th>
<th>Significance Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of Retinoids</td>
<td>6.47</td>
<td>1</td>
<td><strong>0.011</strong></td>
</tr>
<tr>
<td>Condition (either retinoid or vehicle)</td>
<td>4.62</td>
<td>2</td>
<td>0.099</td>
</tr>
<tr>
<td>Concentration x Condition</td>
<td>8.11</td>
<td>2</td>
<td><strong>0.017</strong></td>
</tr>
</tbody>
</table>
Appendix 2:

**Retinoids do not increase the mean length of neurite outgrowth.** The maximal length of neurite outgrowth observed in each condition (DMSO, 9-cis RA, and atRA in the bath) was determined at both concentrations (10^{-6}M and 5x10^{-7}M). Only floating nerves were counted as those submerged do not exhibit outgrowth.

These data were re-analyzed, with the kind help of G. Tattersall, using generalized linear mixed effect models, accounting for the influence of brain “identity” (i.e. which CNS it originated from) as a random effect. Statistical analyses were performed using R software (© The R Foundation). A type-II Wald Chi-square test showed no significant effect of treatment. These data confirmed there was no significant effect of condition on the maximal length of neurite outgrowth of floating nerves at the different concentrations, when nerve identity was accounted for. The findings of this analysis were similar to the findings mentioned previously (on page 47) analyzed using a One Way ANOVA.

<table>
<thead>
<tr>
<th></th>
<th>Chi square value</th>
<th>Degrees of Freedom</th>
<th>Significance Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of Retinoids</td>
<td>1.49</td>
<td>1</td>
<td>0.22</td>
</tr>
<tr>
<td>Condition (either retinoid or vehicle)</td>
<td>0.11</td>
<td>2</td>
<td>0.95</td>
</tr>
<tr>
<td>Concentration x Condition</td>
<td>3.42</td>
<td>2</td>
<td>0.18</td>
</tr>
</tbody>
</table>
Appendix 3:

Description of the retinoid receptor agonists used in this study.

<table>
<thead>
<tr>
<th>Retinoid Receptor Agonist</th>
<th>Chemical Structure</th>
<th>Chemical Name</th>
<th>Alternative Names</th>
<th>Chemical Formula</th>
<th>Molecular Weight (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>atRA</td>
<td><img src="image" alt="atRA Structure" /></td>
<td>3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2E,4E,6E,8E-nonatetraenoic acid</td>
<td>Retinoic Acid Tretinoin</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;28&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>300.44</td>
</tr>
<tr>
<td>9-cis RA</td>
<td><img src="image" alt="9-cis RA Structure" /></td>
<td>3,7-dimethyl-9-(2,6,6-trimethylcyclohexen-1-yl)-2E,4E,6Z,8E-nonatetraenoic acid</td>
<td>9-cis-Tretinoin Alitretinoin</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;28&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>300.44</td>
</tr>
<tr>
<td>EC23</td>
<td><img src="image" alt="EC23 Structure" /></td>
<td>4-(2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)ethynyl)benzoic acid</td>
<td>AGN190205 BASF 46928</td>
<td>C&lt;sub&gt;23&lt;/sub&gt;H&lt;sub&gt;24&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>332.44</td>
</tr>
<tr>
<td>CH55</td>
<td><img src="image" alt="CH55 Structure" /></td>
<td>4-[(1E)-3-[3,5-bis(1,1-Dimethylethyl)phenyl]-3-oxo-1-propenyl]benzoic acid</td>
<td></td>
<td>C&lt;sub&gt;24&lt;/sub&gt;H&lt;sub&gt;28&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>364.47</td>
</tr>
<tr>
<td>SR11237</td>
<td><img src="image" alt="SR11237 Structure" /></td>
<td>4-(2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1,3-dioxolan-2-yl]-benzoic acid</td>
<td>BMS 649</td>
<td>C&lt;sub&gt;24&lt;/sub&gt;H&lt;sub&gt;28&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
<td>380.48</td>
</tr>
</tbody>
</table>
Appendix 4:

Transactivation Activity of RAR/RXR selective retinoids. This table represents the transcriptional activation activity of retinoids for the three subtypes of each RAR and RXR receptor. The half maximal effective concentration (EC\textsubscript{50}) or dissociation constant (K\textsubscript{d}) values were reported from various sources.

The retinoid receptors were obtained from different sources, and different receptor expression vectors and retinoid response element-reporter constructs were used to assess relative transactivation activity. Hence, the transactivation activity for the same retinoids, within a receptor subgroup, may not be the same as that found if all of the retinoids were evaluated in the same assays. Nevertheless, the relative receptor selectivity, compared to other retinoids, should be consistent. In summary, CH55 possess higher affinity for all three subtypes of RAR and EC23 possesses higher affinity for RAR\textalpha and RAR\textbeta, compared to atRA and 9-cis RA. Although SR11237 possess lower affinity for RXR\textalpha compared to 9-cis RA, SR11237 was still found to be an effective activator of RXR\textalpha. Empty cells represent EC\textsubscript{50} or K\textsubscript{d} values that were not determined and NB represents no binding.

<table>
<thead>
<tr>
<th>Retinoid Receptor Agonist</th>
<th>Retinoid receptors</th>
<th>RAR\textalpha</th>
<th>RAR\textbeta</th>
<th>RAR\textgamma</th>
<th>RXR\textalpha</th>
<th>RXR\textbeta</th>
<th>RXR\textgamma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference: Charton \textit{et. al.}, 2009; EC\textsubscript{50} (nm)</td>
<td>EC23</td>
<td>0.6</td>
<td>0.3</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>atRA</td>
<td>17</td>
<td>12</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference: Haffez \textit{et. al.}, 2017; EC\textsubscript{50} (nm)</td>
<td>EC23</td>
<td>3.7</td>
<td>3.3</td>
<td>16.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>atRA</td>
<td>16</td>
<td>17.6</td>
<td>14.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9-cis RA</td>
<td>28.8</td>
<td>27.5</td>
<td>36.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference: Sun \textit{et. al.}, 1997; K\textsubscript{d} (nm)</td>
<td>CH55</td>
<td>0.44</td>
<td>0.04</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>atRA</td>
<td>16</td>
<td>7</td>
<td>3</td>
<td>530</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td></td>
<td>9-cis RA</td>
<td>22</td>
<td>11</td>
<td>20</td>
<td>8.4</td>
<td>7.4</td>
<td>13</td>
</tr>
<tr>
<td>Reference: Dawson \textit{et. al.}, 1995; EC\textsubscript{50} (nm)</td>
<td>SR11237</td>
<td>55</td>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>atRA</td>
<td>54</td>
<td>4.5</td>
<td>2</td>
<td>530</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9-cis RA</td>
<td>23</td>
<td>2.6</td>
<td>4.3</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Appendix 5:**

Methods for measuring the proportional area occupied by regenerative processes (%) in ImageJ software. Highlighted in bold are the menu bar options and tools clicked in ImageJ.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>The ImageJ program was opened</td>
</tr>
</tbody>
</table>
| (2) | **File** → **Open** → Images of regenerative processes were chosen from folder  
Note: All images had been taken at the same magnification (10x) |
| (3) | Images were spatially calibrated so that distances measured in the image were measured in µm, as shown below:  
**Analyze** → **Set Scale** → Values were adjusted as below then **OK** was clicked: |
| (4) | Next, to ensure the percent of area occupied by regenerative processes (%) was measured within the same specified area, a circle with an area of 135620 µm² was drawn and saved into the ImageJ program. This allowed for this same “circle” (of equal area) to be retrieved for conducting measurements in each image. The “circle” was drawn and saved into the ImageJ program as follows:  
**Plugins** → **Macros** → **Record**  
**Oval Selection Tool** was chosen → A circle was drawn → **Analyze** → **Measure**  
The area displayed was 135620 µm²  
Next, the **Create** option was clicked in the Recorder tab → **File** → **Save as** → The circle was saved as a file on the computer to be easily retrieved for use afterwards |
To retrieve the circle:
**Plugin → Marcos → Run** → The file name of the circle that it had been saved as was chosen

(5) The circle was then moved, using the mouse, such that the circle encompassed the neurite outgrowth and the center of the circle aligned with the center of the cut nerve ending.

(6) The neurite outgrowth was highlighted in white and the background was eliminated, to allow for measuring the percent of area occupied by only the neurite outgrowth (%) within the specified area. This was conducted as mentioned below:

**Process → Sharpen**

**Process → Find Edges**

**Image → Adjust → Threshold → Apply**

(7) Finally, the area occupied by the neurite outgrowth within the specified circle was measured:
**Analyze → Measure** → The value recorded under **Percent Area** represented the percent of area occupied the neurite outgrowth highlighted in white (%)
References


Nuclear Receptor Expression in Human Pancreatic Carcinoma Cell Lines. Gastroenterology


Saito, Y., Okamura, M., Nakajima, S., Hayakawa, K., Huang, T., Yao, J., and Kitamura, M.
(2010) Suppression of nephrin expression by TNF-alpha via interfering with the cAMP-retinoic


104: 55–62.


depression at both in vivo and in vitro reconstructed synapses between identified Lymnaea

Song, H.J., Ming, G.L., and Poo, M.M. (1997) cAMP-induced switching in turning direction of

Song, H., Ming, G., He, Z., Lehmann, M., McKerracher, L., et. al. (1998) Conversion of
neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. Science
281:1515–18.

regulates cell and organism size during mouse embryonic development. Dev. Cell, 2: 553–565

Differential effects of synthetic nuclear retinoid receptor-selective retinoids on the growth of


Syed, N., Bulloch, A., Lukowiak, K. (1990) In vitro reconstruction of the respiratory central


