A possible role for chemokine signalling through CXCR4 as a downstream effector of retinoic acid signalling in the regenerating tail and spinal cord of *Notophthalmus viridescens*

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Abstract

The newt, *Notopthalmus viridescens* is one of the few tetrapod vertebrates capable of extensive regeneration of the central nervous system, however, the factors involved in this process are still unknown. Chemokine signalling through the receptor CXCR4, has been found to be involved in the development of the central nervous system of mammals and more recently in epimorphic fin regeneration in zebrafish. We have hypothesized that the CXCR4 signalling pathway is involved in spinal cord and tail regeneration in the adult newt, possibly as a downstream target of retinoic acid signalling. We found that CXCR4 mRNA expression was observed in the brain, spinal cord, heart, gut, liver and regenerating tail blastemas. CXCR4 expression increased over the first 12 days of tail regeneration and returned to basal expression levels at day 21 of regeneration. Inhibition of CXCR4 with AMD3100, a specific receptor antagonist, led to a decrease in CXCR4 mRNA in the regenerating tail 14 days post amputation. Histological analysis suggests a delay in the early stages of tail and spinal cord regeneration. Spinal cord explants treated with CXCL12, the ligand to CXCR4, displayed enhanced neurite outgrowth *in vitro*. Explants treated with AMD3100 abolished any retinoic acid enhanced neurite outgrowth effects suggesting a link between these signalling pathways.

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Chapter 1: Introduction and Objectives

1.01 Introduction

The mammalian central nervous system (CNS) in adulthood is very limited in its regenerative ability after injury or disease. Extensive CNS regeneration in mammals is limited during development in the prenatal period of life. Urodele amphibians such as *Notophthalmus viridescens*, more commonly known as the red spotted newt, are the only tetrapod vertebrates that are strong regenerators of the CNS into adulthood (Chernoff et al., 2003). Unfortunately there are large gaps in our understanding of this remarkable biological process.

Regeneration studies in urodeles have demonstrated that ependymal cells lining the central canal of the spinal cord are capable of producing functional neurons (Benraiss et al., 1998), in a fashion similar to mammalian neural stem cells (Zhang et al., 2002). The proliferating cells from the ependymal tube differentiate into a variety of cells which include neurons. The neurons eventually send axonal projections through the newly reformed SC and then to their respective targets (Chernoff et al., 2003).

One of the key promoting factors of regeneration discovered thus far is retinoic acid (RA), a metabolite of vitamin A. During development, RA is involved in patterning and neuronal differentiation. If RA is absent, the hindbrain does not develop, and the spinal cord develops abnormally (Maden, 2007). In the embryonic vertebrate CNS, where regeneration is possible, injury results in the upregulation of retinoic acid receptor beta (RARβ),(Maden, 2001). Viral vector-mediated overexpression of RARβ2 in the adult rat spinal cord is able to promote the regeneration of functional sensory axons

(Wong et al., 2006). It has also been demonstrated that RA acts as a chemoattractant for regenerating adult newt spinal cord axons *in vitro* (Dmetrichuk et al., 2005). Unfortunately the downstream targets of the transcription factor RARβ2 are not well known at this time. Microarray studies have identified a number of potential target genes that are up or down regulated by injury to the spinal cord. In the adult mammalian SC, some of these genes are regulated (up or down) in the opposite direction of RARβ2, if RARβ2 is constitutively expressed from a lentiviral vector. One such gene is the chemokine receptor, CXCR4 (Wong, Personal Communication)

Chemokines were originally discovered due to their roles in the immune system (Baggiolini et al., 1994). Recently, chemokines have also been discovered to play diverse roles in development, homeostasis, angiogenesis, tumour progression and metastasis. In the central nervous system, chemokines are able to act as repellent or attractive cues for growing/regenerating axons (Bajetto et al., 2002; Opatz et al., 2009). The most recent discovery that CXCR4 may be a potential downstream target of RARβ2 during spinal cord regeneration raised intriguing research possibilities.

1.02 Objectives

My hypothesis was that CXCR4 signalling is involved in tail and spinal cord regeneration in *N. viridescens* and is associated with RARβ signalling. My objectives were as follows: 1) Obtain the full length newt CXCR4 cDNA sequence and characterize the expression pattern of CXCR4 in the newt during tail and spinal cord regeneration. 2)

Investigate if CXCR4 is functionally involved in spinal cord regeneration in the tail. To do this I inhibited CXCR4 signalling and investigated any changes in a) the temporal and spatial expression pattern of CXCR4 and b) the gross morphology and histology of tail and spinal cord regenerates. 3) Conduct *in vitro* spinal cord explant studies to determine if CXCL12, the natural ligand of CXCR4 could enhance neurite outgrowth. 4) Investigated if inhibition of CXCR4 signalling *in vitro* could block the neurite outgrowth promoting effects of retinoic acid in adult newt spinal cord explants. Results from these studies provide evidence that CXCR4 signalling is involved in regeneration and that a functional link exists between retinoid and chemokine signalling pathways in adult newt spinal cord regeneration.

Chapter 2: Literature Review

2.01 Regeneration

The biological process of regeneration has intrigued researchers for well over two centuries since it was first documented by Spallanzani in 1768. Regeneration is commonly defined as the functional regrowth of a body structure, organ, or tissue that was lost or injured. Urodele amphibians are the only tetrapod vertebrates capable of extensive regeneration of appendages such as the limb and tail, and various organs and tissues throughout their lifetime (Ferretti et al., 2003). Epimorphic regeneration refers to the regrowth of a body structure, such as a limb. The process of epimorphic regeneration in the adult newt limb and tail differ slightly, and will be discussed later. The regeneration process of specific tissues within the tail such as the spinal cord will be examined separately.

2.02 Tail Regeneration

Amphibian tail regeneration can be divided into three major steps: 1) wound healing, 2) blastema formation and proliferation, and 3) outgrowth and patterning (Iten and Bryant, 1976; Singer et al., 1979). A few hours after amputation of the tail, epithelial cells from the stump migrate and cover the wound surface and form what is called the wound epithelium. Unlike wound healing in mammals, it is important to note that the dermis does not travel with the epidermis, which prevents the formation of a scar. The cells proliferate and form a wound epithelium similar to the apical ectodermal cap in limb regeneration (Chernoff and Stocum, 1995). Next, the cells of the tissues in close proximity to the wound epithelium appear to dedifferentiate and form a mass of

undifferentiated cells. Dedifferentiation is the process whereby specified cells revert back to a multipotent undifferentiated state (Liu et al., 2006). This mass of undifferentiated cells is referred to as the blastema. These cells will continue to proliferate and eventually redifferentiate and contribute to forming most of the tail structure. It is important to note that complete tail regeneration also requires the regeneration of the nervous system, especially the caudal spinal cord, to proceed normally (Chernoff et al., 2003).

Cell tracing studies have helped us to more clearly understand the origin of blastema cells and the differentiation and redifferentiation processes. The two major tissues that have been extensively studied are muscle and neural tissue, which are mesodermal and ectodermal in origin. Lo et al. (1993) confirmed previous studies that muscle fibres near the amputation site in the forelimb dedifferentiate. This was achieved by transplanting differentiated rhodamine-dextran-labeled myotubes into the amputated newt limb. They discovered that the blastema contained labelled, dedifferentiated cells. Changes in cell fate were also observed where muscle cells gave rise to cartilage. The process of transdifferentiation has also been documented.

Transdifferentiation refers to the process where cells revert to a dedifferentiated state and redifferentiate into another cell type from a different germ layer. Echeverri and Tanaka (2002) reported that spinal cord cells migrated and formed muscle and cartilage, confirming that cell line switching from ectoderm to mesoderm occurred in axolotl tail regeneration. It is not yet known if transdifferentiation from mesoderm to

ectoderm occurs during tail regeneration. These processes of dedifferentiation and transdifferentiation are typical of urodele regeneration.

More recently, Kragl et al. (2009) reported that the extent of cellular dedifferentiation during regeneration may not be as great as previously thought. Looking at axolotl limb regeneration, they discovered that each tissue of the amputated limb produced a population of cells with restricted potential. This produced a limb blastema comprised of heterogeneous cells, which is very different from what was previously thought. This also indicates that regeneration can occur without cells returning to a fully dedifferentiated pluripotent state (Kragal et al., 2009). It is not yet known if tail regeneration in the axolotl or other regenerating animals such as the newt undergo a similar restricted dedifferentiation process.

There are various differences in the epimorphic appendage regeneration process between anurans and urodeles. For instance, a structural equivalent of a true blastema is not seen in anuran regeneration. In *Xenopus* tadpoles undergoing tail regeneration, undifferentiated cells with limited potency are found in what resembles a blastema. Also, cell lineage is not changed during *Xenopus* tail regeneration (Ryfell et al., 2003; Slack et al., 2004).

The role of neural tissue is also of great importance to regeneration. The peripheral nervous system and the central nervous system are both present in the tail, and their regeneration is crucial for tail regeneration to proceed normally. Normal regeneration in the limb requires innervation by brachial nerves (Singer, 1952).

Denervation studies show that regeneration is impaired if nerves are severed. Tail regeneration is also a nerve dependent process and is greatly dependent upon spinal cord regeneration (Holtzer, 1956). These processes will be covered in greater detail in the following section.

2.03 Spinal Cord Regeneration

The regenerative ability of the spinal cord in adult urodeles is unique and also puzzling. Other vertebrates such as mammals do not have the same ability to regenerate the CNS in adulthood. Furthermore, it appears that there are a variety of factors and events that prevent regeneration in most vertebrates. Some of these are inhibitory factors produced from the breakdown of myelin, excitotoxicity from calcium influx, and glutamate toxicity (Chernoff and Stocum, 1995). The formation of an astroglial scar and cavitation also produce physical barriers that prevent spinal cord regeneration in most vertebrates (Yiu and He, 2006). Neuroinflammation also causes further degradation to the damaged central nervous system. This area of research has received a great deal of attention. It is believed that the immune response is necessary after injury or infection to limit further damage, however theories are beginning to emerge that the immune system can be enhanced or altered to promote CNS repair (Popovich and Longbrake, 2008). Interestingly, the immune response of animals with regenerative abilities differs slightly from those that are unable to regenerate.

The urodele amphibian response to spinal cord injury differs markedly from that of mammals. The myelin of the urodele CNS does not appear to have an inhibitory effect

toward axonal outgrowth, however myelin in the adult *Xenopus,* which cannot regenerate the CNS extensively, does have an inhibitory effect (Lang et al., 1995). Next the injured urodele spinal cord does not form a glial scar as it does in mammals. Also, there is an "ependymal response" that promotes spinal cord regeneration after cord transection or tail amputation. Shortly after tail amputation, the spinal cord retracts and is quickly sealed off by the population of ependymal cells that form the ependymal bulb. The ependymal cells of urodeles are unique in that they remain in contact with the pia mater throughout adulthood and resemble radial glia (Chernoff et al., 2003). The ependymal cells lining the central canal of the spinal cord begin to undergo important changes such as the upregulation of nestin expression, which is considered to be a neural cell progenitor marker (Ferretti et al., 2003). Thus during axolotl spinal cord regeneration the proliferation of ependymal cells produces a population of neural progenitors (Egar and Singer, 1972). It is theorized that these ependymal cells undergo some form of activation process which is part of the initiation process for regeneration. These cells that act as neural progenitors during regeneration possess similar characteristics to embryonic progenitors which have been found in the mammalian CNS (Doetsch et al., 1999). Therefore it may be possible in the future to induce adult progenitor cells in mammals to promote regeneration.

After the ependymal cells are activated in adult newts, they continue to proliferate and form an ependymal bulb and a terminal vesicle at the distal tip of the newly regenerating spinal cord. Eventually an ependymal tube, which now resembles a

developing neural tube begins to extend from the spinal cord into the tail blastema. Elongation occurs via proliferation. Dividing cells, as evidenced by mitotic figures, are most frequently seen along the ependymal tube and are less common at the terminal vesicle (Iten and Bryant, 1976). Eventually the ependymal cells begin to differentiate in a proximal to distal direction. Cell labelling studies using bromodeoxyuridine (BrdU) allowed Benraiss et al. (1999) to determine that new neurons and glial cells in the regenerating tail originated from the proliferative ependymal layer of the ependymal tube. Egar and Singer (1972) reported that dendrites and axons grow between the ependymal processes and stay in close contact with the basal lamina. The literature review to this point covers the fundamental process of regeneration during the early stages of regeneration (Stages I –III), outlined by Iten and Bryant (1976). Stage IV and V mainly consists of growth and further differentiation of cells and tissues (Iten and Bryant, 1976). Now that I have outlined the morphological features of early regeneration, is it critical to examine the factors that may act on these tissues during the early stages of regeneration.

2.04 Factors which Promote Regeneration

There are many theories as to why certain vertebrates are able to regenerate various tissues and appendages (Tanaka and Ferretti, 2009). Some of these include retention of embryonic traits (neoteny), retention of stem and progenitor cells, and a high degree of cellular plasticity, which gives the cells the ability to reactivate developmental pathways in adulthood. Interestingly, neoteny, which is seen in the

axolotl and many believe contributes to its regenerative abilities, is not present in newts, however newts are capable of extensive regeneration. Thus, the process of regeneration and the factors which promote regeneration varies greatly among species and sometimes within the same species, depending on the location or type of injury (Tanaka and Ferretti, 2009). Further evidence supports the theory that neoteny may be involved in the ability to regenerate into adulthood for some animals because many vertebrates, including mammals, are capable of some form of regeneration during embryonic stages of life.

It is still not well known to what extent developmental pathways are recapitulated during regeneration in adult organisms. Spinal cord regeneration in adult newts does not mirror all events of neural development. However various signalling pathways are reactivated. Holtzer (1956) found that by rotating a piece of tail spinal cord by 180° about the dorsal ventral (DV) axis and implanting it back into the tail, the surrounding tissue regenerated upside down (inversion of the DV axis). This observation suggests that the spinal cord in urodeles retains DV patterning information which is transmitted to the regenerating tail when needed. Some developmental marker genes that have been identified during tail regeneration in axolotls include *Pax6, Pax7, Msx1* and *Sonic hedgehog (Shh)* (Schnapp et al., 2005). All markers were expressed in the mature spinal cord, regenerating spinal cord and ependymal tube in the DV domain in a fashion very similar to what is seen during development. *Shh* was found in the ventral cells of the spinal cord, which would be analogous to the floor plate in development.

Pax6 was found in the lateral parts of the spinal cord and *Pax7* was found more dorsolaterally. Finally *Msx1* was found in the dorsalmost part of the spinal cord that resembles *Msx1* expression from the roof plate during development. It was also reported that the *Shh* receptor *Patched* was not only expressed in the regenerating spinal cord/ependymal tube, but also in the blastema. It was also discovered that *Shh* regulates the proliferation of the blastema and not ependymal cell proliferation. Therefore *Shh* is necessary for blastema growth and regeneration but is not sufficient for tail regeneration. It is important to note here that chemokine signalling through CXCR4 is also involved in guiding proper motor axon trajectories in the developing mammalian neural tube (Liberam et al., 2005).

2.05 Cellular Organization of the Regenerating Spinal Cord and Tail

The origin of neural progenitor cells has also been an area of interest in regeneration. There are two main theories which explain where progenitors originate; 1) activation of stem cells, 2) dedifferentiation. Dedifferentiation is the process whereby a cell regresses to an earlier undifferentiated stage, re-enters the cell cycle, and then redifferentiates. Less information is available about this theory regarding regeneration in the spinal cord than the limb and tail. It is thought that the ependymal cells, which possess a high degree of plasticity, undergo dedifferentiation during regeneration. There is also evidence that these cells are activated to become neural progenitors suggesting that these two processes may be equivalent (Ferretti et al., 2003).

Evolutionary changes also appear to correlate with regenerative abilities in animals. Two major factors that determine the success of regeneration in vertebrates appear to be the complexity of the nervous system, and immune system. The appearance of myelin in vertebrates correlates with a reduction in regenerative abilities when compared to invertebrates. Animals with more "advanced" or complex nervous systems, possessing a neocortex, generally do not regenerate. Newts, which are considered to be "lower vertebrates" that do not have a neocortex, have a greater ability to regenerate than do "advanced" vertebrates such as ourselves (Popovich and Longbrake, 2008).

With respect to immunity, primitive animals that have greater regenerative abilities, have only a primitive form of innate immunity. In response to injury, there is little or no inflammatory response, which is quite different from the CNS of an injured mammal (Mescher and Neff, 2005).

2.06 Factors which Limit or Prevent Regeneration

As previously stated, most vertebrates do not readily regenerate their CNS after injury or disease. Here I will outline the events that cause further damage to the spinal cord in non-regenerating vertebrates such as mammals and will discuss other mechanisms that prevent regeneration. Blunt trauma or other forms of mechanical injuries that crush or sever the spinal cord are known as primary injuries. After the primary injury, a series of events take place that cause more damage to the spinal cord and are referred to as secondary injuries. Events that result in secondary injuries include

ischemia, free radical damage, excitotoxicity, necrotic cell death and inflammation (Tator and Fehlings, 1991). The same mechanical trauma can cause similar primary injuries to regeneration competent animals such as newts, however based on the review of the literature, secondary injuries seem to be avoided or greatly reduced.

Damage to the vasculature results in haemorrhaging that greatly reduces the supply of nutrients to the spinal cord, as well as disposal of metabolic waste products. The major consequence is ischemia (Tator and Fehlings, 1991). Due to the metabolic demands of the cells in the spinal cord, they are quite sensitive to ischemia, resulting in necrotic cell death. Damage caused by reperfusion is also common, due to the build up of oxygen-derived free radicals (Lukanova et al., 1996). Free radicals are known to cause damage to the cell membrane, DNA, and many proteins, all of which are detrimental to the cell. These events most likely result in further necrotic and apoptotic cell death (Cuzzocera et al., 2001). Ischemia, which is also common after vascular damage, has been observed to impede regeneration. Tassava and Huang (2005) experimentally produced extensive ischemia in regenerating newt tails and found that regeneration was impaired.

Excitotoxicity, mainly caused by the uncontrolled release of glutamate from damaged neurons, also results in secondary injury. The glutamate receptor N-methyl D-aspartate (NMDA) has been studied because of its role in regulating calcium in the cell. If NMDA is activated, extracellular calcium moves into the cell, and may also trigger the release of intracellular calcium stores (Mody and MacDonald, 1995). Many cellular

processes are calcium dependent and the improper activation of these processes can lead to further necrosis (Choi, 1987).

2.07 The Role of the Immune System in Regeneration

The immune response in the CNS is also quite important after spinal cord injuries. In higher vertebrates, it involves the cellular components such as macrophages, neutrophils, T cells, and non cellular components such as prostaglandins and cytokines (Popovich et al., 1997). There is some controversy regarding the role of the immune system as to whether it helps to protect the CNS or inadvertently causes further damage. The immune system has neuroprotective properties but also neurotoxic side effects (Bethea et al., 1999). For example the cytokine, tumour necrosis factor alpha (TNF-α), has been shown to increase the inflammatory response resulting in increased cell damage. It has also been shown to be neuroprotective against cell death (Bethea et al., 1999).

Along with myelin having inhibitory properties, the astroglial scar that eventually forms around the injury site, further inhibits any recovery or regeneration. After injury in mammals, reactive astroglia proliferate around the injury site. Next, the reactive astrocytes induce the synthesis of chondroitin sulphate proteoglycans, which have potent inhibitory properties against neurite outgrowth *in vitro* and *in vivo* (Bush and Silver, 2007). The astroglial response in maintaining homeostasis in the CNS aids greatly in neuroprotection, however in the context of traumatic injury, wound healing or regeneration, its effects are seen as negative. As with many cellular responses, the

astroglial response is regulated by a variety of neuroimmune regulatory proteins. These regulatory proteins are produced by neurons, glia, and the ependyma. For instance, signalling through the chemokine CX3CL1 (Fractalkine) and its receptor CXCR1, which is mainly found on microglia, restricts the extent of microglia activation and reduces the neurodegeneration and scarring after neural damage (Cardona et al., 2006).

Mescher and Neff (2006) provide a comprehensive review that discusses how organisms that can undergo epimorphic regeneration, avoid the inhibitory effects of an inflammatory response after traumatic injury. This local suppression of the immune response or "peripheral self-tolerance" is believed to be a key factor that allows organisms such as newts to regenerate. Injuries in regenerating animals induce a suppressed immune response, where lymphocytes are eliminated and the production of lymphocytes in the thymus is suppressed until regeneration is complete. Also, regenerating animals demonstrate poor adaptive immunity, which correlates with increased regenerative abilities (Mescher and Neff, 2006)

2.08 Chemokines and Receptors

Chemokines were originally discovered due to their roles in the immune system. They are similar to cytokines and have chemotactic effects but are smaller and signal mostly through G coupled protein receptors. Baggiolini et al., (1994) reported that Interleukin 8, (now referred to at chemokine CXCL8) and other related small chemotactic cytokines were able to direct the migration of immune cells to sites of inflammation. Aside from their roles in leukocyte communication and migration,

chemokine signalling plays an important role in acute and chronic inflammatory responses. Interruption in chemokine expression and signalling has been associated with pathological inflammatory conditions such as arthritis, glomerulonephritis, inflammatory bowel syndrome, and atherogenesis (Baggiolini et al., 2001). Chemokines also play important roles in homeostasis, angiogenesis, and cancer progression, but their roles during development, particularly in the CNS, are the most relevant to this review and will be discussed in more depth.

Chemokines are secreted molecules that range from 8 – 14 kDa in size and are classified based on the position of the first N-terminal cysteine residues into four known groups; C, CC, CXC, and CX3C. The α-chemokines (CXC) and the β-chemokines (CC) are the two largest groups. δ-chemokines (CX3C) consist of only one member, Fractalkine/CX3CL1, whose function in neuroinflammation has already been discussed.

Chemokines signal through their corresponding receptor, which is classified based on which ligands they bind to. These molecules display promiscuous binding but some monogamous chemokine binding interactions have been suggested. CXCL12/CXCR4 was originally thought to be monogamous but recent work has shown that CXCR7 can also bind CXCl12 (Balabanian, 2005). More recently, Levoye et al., (2009) have shown that CXCR4 heterodimerizes with CXCR7, which modulates signalling through various G protein subunits, explaining the functional plasticity of its ligand, CXCL12.

Chemokine receptors belong to the superfamily of seven-transmembrane domain receptors, which signal through heterotrimeric G-protein subunits. Chemokine signalling works through many pathways, some of which include phospholipase C, Ca^{2+}, and JAK/ STAT pathways and is reviewed in Bajetto et al. (2002). Here, we will focus on the G protein coupled receptor (GPCR) CXCR4 and signalling most relevant to the nervous system during development and regeneration. CXCR4 is widely expressed throughout the nervous system and is responsible for multiple cellular responses in a variety of cells. To better understand the roles of CXCR4 and its signalling pathways, we will examine the main cellular responses (cell migration, proliferation, cell survival, axon guidance) in specific contexts.

2.09 CXCR4 Signalling During Cell Survival, Proliferation, and Migration

Chemokine signalling has been shown to regulate cell proliferation and survival during the development of the CNS. *In vitro* studies have shown that the proliferation of glial cells is regulated by CXCL12/CXCR4 signalling. Astrocyte proliferation through ERK1/2 activation has also been observed to be induced by CXCL12/CXCR4 signalling. In many cell types, proliferation signals involve extracellular signal-related kinases (ERKs). Bajetto et al. (2001) demonstrated that CXCL12-induced proliferation of astrocytes was dependent on the activation of the ERK1/2 pathway. Also astrocyte proliferation was inhibited by pertussis toxin (PTX) and wortmannin, indicating the involvement of PTX sensitive G-protein and phosphatidyl inositol-3-kinase signalling. These pathways are not only involved in proliferation but also in cell survival.

During the development of the cerebellum in mice, CXCL12/CXCR4 and Sonic hedgehog (SHH) synergistically promote granule cell proliferation in a pertussis toxin sensitive manner (Klein et al., 2001). These granule cells (immature cerebellar cells) are located in the external growth layer that is directly under the pia mater of the CNS. Immunohistochemistry showed that CXCL12 was present in the pia matter whereas CXCR4 was present in the proliferating granule cells (Klein et al., 2001). This complementary expression pattern of ligand and receptor is seen in a variety of tissues utilizing the CXCL12/CXCR4 signalling pathway.

Cell migration is a widespread result of CXCL12/CXCR4 signalling, particularly in the immune system. However I will focus on the role of chemokine signalling in the migration of neural progenitor cells and glia. Major changes during cell migration require changes in the expression of specific cell adhesion molecules and re-modeling of the cytoskeleton through activation or deactivation of certain actin binding proteins. Using neural stem cells from mouse and human lines, Imitola et al. (2004) showed that the migration of neural stem cells to sites of CNS injury are directed by the CXCL12/CXCR4 pathway. CXCL12 promoted migration of neural precursor cells (which express CXCR4) and enhanced proliferation. Signal transduction pathway components that are thought to be involved in migration include c-jun, p38MAPK, and paxillin. These pathways are thought to converge on the cytoskeleton during migration. Chemotaxis mainly requires the formation of an internal gradient of Ca^{2+} and G proteins such as

CXCR4. The details regarding signalling pathways initiated by CXCR4 signalling in the nervous system have only begun to be elucidated.

Luo et al. (2006) reported that CXCL12/ CXCR4 signalling in neural progenitor cells stimulated β-catenin transcriptional activity through the CXCR4 G protein receptor mediated ERK activation pathway. Evidence of cross talk between the Wnt and CXCR4 pathways was also shown. It was also proposed that interactions with β-catenin, cell adhesion molecules, and microtubule complexes are likely to underlie the CXCl12 modulated migration and axon guidance.

Cell migration and chemotaxis in the immune system provide a better understood model system for understanding the role of CXCL12/CXCR4 signalling. Nishita et al. (2002) reported that CXCL12 activates LIM kinase 1 which phosphorylates cofilin, promoting the reorganization of the actin cytoskeleton in T-cells. The Rho family of small GTPases also play various roles in cytoskeleton dynamics. Here, LIM kinase 1 activation was found to be mediated by Rac.

Some events where CXCL12/CXCR4 dependent migration occurs, but the specific signalling pathways have yet to be defined, include gastrulation, development of the nervous system, and migration of cells to neuroinflammation. During frog gastrulation, CXCL12/CXCR4 signalling is necessary for the migration of many cells (Fukui et al., 2007). CXCR4 is expressed in the involuting marginal zone and complemented by the expression of CXCL12 on the inner surface of the blastocoel roof. Disruption of CXCL12/CXCR4 signalling resulted in gastrulation defects and it was also demonstrated

that the leading edge of the involuting mesoderm was directed towards sources of CXCL12 (Fukui et al., 2007).

GABAergic neurons of the cortex originate in the ventral telencephalon and migrate via tangential migration. In CXCR4 -/- or CXCL12-/- mice, GABAergic neurons fail to migrate to their appropriate targets. It was found that CXCL12 was expressed along the migration path of GABAergic neurons and that in postnatal rat pups, the GABAergic cells no longer expressed CXCR4 (Stumm et al., 2007).

Neural stem cells also exhibit migratory abilities linked to CXCL12 expression. Takeuchi et al. (2007) intravenously transplanted neural stem cells and found that they migrated to injury sites in the adult mouse spinal cord. The migration of the cells correlated with peak expression of CXCL12 mRNA in the lesion at 7 days after injury. The transplanted cells eventually differentiated into neuronal and glial cells 21 days after being transplanted. CXCL12 and other various chemokines such as CCL2 and their various receptors also have regulatory roles that direct neural progenitors to sites of neural inflammation. Belmadani et al. (2006) demonstrated that chemokines are secreted by sites of neuroinflammation and are attractive to neural progenitor cells.

2.10 CXCR4 Signalling and Axon Guidance

The development and regeneration of the CNS also requires proper synaptic connections to be made. A variety of guidance cues help to guide axonal growth cones to their appropriate target sites (Tessier-Lavigne and Goodman, 1996). New evidence

has arisen that CXCL12/CXCR4 is also involved in axonal guidance. Xiang et al. (2002) found that growth cones of cerebellar granule cells from rats turned away in response to a gradient of CXCL12. The response was mediated by G_i, a G protein subunit that activates phospholipase C, which subsequently activates two more pathways: protein kinase C (PKC) and inositol 1,4,5-triphosphate (IP_3). PKC signalling was found to be repulsive and was the dominant effect. Elevating levels of cytosolic cyclic nucleotides such as cyclic GMP could convert repulsive turning into attraction. Thus GPCRs such as CXCR4 could regulate both repulsive and attractive axon guidance.

CXCL12/CXCR4 signalling has also been shown to be involved in guiding retinal ganglion cells in zebrafish (Chalasani et al., 2007). The expression of CXCL12 was shown to make retinal ganglion cells less sensitive to the repulsive effects of slit2, thus modulating guidance cues. Evidence also suggests that an elevation of intracellular cAMP is also involved in modulating axon guidance (Chalasani et al., 2007). Liberam et al. (2005) found similar results regarding the trajectory of ventral motor neurons from the developing neural tube in mice. Here, in CXCL12(-/-) and CXCR4 (-/-) knockout mice, ventral motor neurons displayed aberrant projections when exiting the neural tube and adopted a fate that resembled dorsal motor neurons. Interestingly, dorsal motor neurons do not express CXCR4. It is thought that CXCR4 is involved in axon guidance by making the ventral motor neurons insensitive to the repellent cues that are present at the ventral exit point of the neural tube, thus working in a similar fashion to modulating repellent cues in zebrafish axon guidance. It is important to note that CXCR4 signalling

does not always have the same effect. The response of CXCR4 has been also shown to be dependent on neural cell type and can either promote, or inhibit axon outgrowth (Li and Ransohoff, 2007).

Arakawa et al. (2003) found that different levels of CXCL12/CXCR4 signalling could either promote extension or inhibit extension of axons from cerebellar granule cells of rats. This was found to be the result of a concentration dependent activation of Rho signalling pathways. Low level CXCL12 signalling increased axon elongation, but higher concentrations of CXCL12 activated the Rho/ROCK pathway and repressed axon elongation. Here we find that CXCL12/CXCR4 can signal through various Rho-GTPases, further expanding the diverse signalling abilities of CXCR4.

CXCL12/CXCR4 signalling is also involved in the development of the olfactory system in mammals (Toba et al., 2008). Various CNS neurons, such as gonadotropin releasing hormone-1 (GnRH-1) neurons, migrate from the nasal region into the developing brain and are CXCR4 immunopositive. CXCR4 deficient mice showed a decrease in GnRH-1 neurons. Signal inhibition by the CXCR4 specific inhibitor AMD3100 prevented GnRH-1 neuronal migration and sensory axon outgrowth *in vitro.* They concluded that CXCL12/CXCR4 expression was not necessary for axon guidance, but influenced neuronal extension and migration (Toba et al., 2008)

2.11 CXCR4 and Regeneration

The involvement of CXCL12/CXCR4 signalling has been implicated in a variety of injury and regeneration models, such as the repair of cardiovascular tissue injury (Napoli et al., 2007). A well studied system in which CXCL12/CXCR4 signalling is involved in regeneration of adult structures is that of the zebrafish fin (Dufourq et al., 2006). CXCL12 and CXCR4 expression is not seen in the unamputated fin. CXCL12 expression was first detected in the fin blastema at 2 days post amputation, peaked after 3 days and then was downregulated by day 5. Various isoforms of CXCR4 have also been identified in zebrafish fin regeneration. CXCR4a expression peaked at day 3 and was localized to the fin stump. The expression of CXCL12 and CXCR4a is transient and any modulation of CXCL12 activity completely abolishes fin regeneration (Dufourq et al., 2006). More recently it was discovered that CXCL12/CXCR4 signalling is regulated by Fgf signalling (Bouzaffour et al., 2009). Fgf signalling activates the expression of CXCL12 in the fin blastema, which then activated the expression of CXCR4a in the stump epidermis, and CXCR4b in the wound epithelium, while inhibiting CXCR7. It is thought that CXCL12 plays two major roles in fin regeneration. The first is to mediate Fgf activity and aid in the proliferation of epithelial cells. The second is to turn off Fgf activity possibly in cooperation with Wnt signalling. These examples of CXCR4 signalling are not an exhaustive overview, but only a focused review of mechanisms most relevant to epimorphic regeneration of the urodele tail and spinal cord.

2.12 Retinoids, CNS development and Regeneration

Many factors have recently been shown to promote functional regeneration of the adult central nervous system. The most relevant from this review is retinoic acid (RA), and signalling though one of its receptors, retinoic acid receptor beta (RARβ). RA is a naturally occurring vitamin A metabolite and has two major roles in the developing nervous system that are well defined. The first is as a patterning factor where it contributes to the anteroposterior and dorsoventral patterning of the developing hindbrain and spinal cord (Maden, 2002). The second role that RA plays is in neuronal differentiation. *In vitro*, RA can induce the differentiation of neurons and glia through the activation of various genes, including those encoding transcription factors, receptors, and signalling molecules (Maden, 2001).

During RA signalling, RA is first released in a paracrine fashion and taken up by the receiving cells. Autocrine signalling can also occur, but in both cases RA must enter the nucleus with the assistance of a cellular retinoic acid binding protein, CRABP2. Next, RA binds to a transcription factor complex that is made up of two ligand activated transcription factors, the RA receptor (RAR) and the retinoic X receptor (RXR). The heterodimeric complex binds to a DNA sequence known as the retinoic acid response element (RARE). Aside from ligand binding, co-activators, co-repressors, and phosphorylation of the receptors are required for the activation or repression of gene transcription (Maden, 2007).

RA signalling has also been shown to be involved in CNS regeneration. In the adult mammalian nervous system, RARβ2 is not normally expressed, nor does spinal cord injury induce the expression of RARβ2 in mammals (Cororan et al., 2002). Yip et al. (2006) found that by increasing the expression of RARβ2 in the adult rat spinal cord, functional recovery from corticospinal tract injuries was promoted. This was accomplished by using a lentiviral vector that expressed RARβ2. Injected rats showed an increase of RARβ2 expression and an increase in axonal projections in and beyond the damaged corticospinal tract. Finally, rats displayed functional recovery of both motor and sensory behaviours at 6 weeks post lesion.

RA has been shown to have chemotropic effects on regenerating limb blastemas and spinal cord explants from *N. viridescens* (Dmetrichuk et al., 2005). RA was shown to enhance and also direct axonal outgrowth from cultured spinal cord explants *in vitro*. Endogenous RA was also found in the regeneration limb blastema. When blastemas were co-cultured with spinal cord explants, the blastemas induced more axonal outgrowth on the side of the explant nearest to the blastema (Dmetrichuk et al., 2005). With regard to spinal cord regeneration in the tail, RARβ2 expression is low in the undisturbed spinal cord. At 8 days post amputation of the tail and spinal cord, RARβ2 expression is greatly increased (Carter et al., 2006). It is believed that this endogenous ability to upregulate RARβ2 expression in the adult newt is one of the key factors that promote functional regeneration in this urodele amphibian.

Unfortunately the downstream targets of RARβ2 signalling are not yet well defined. Microarray analyses have identified a variety of genes whose expression is altered in response to ectopic RARβ2 in the injured mammalian spinal cord (Maden, personal communication). Alterations in the expression of regeneration associated genes suggests that they may be downstream regulatory targets of retinoid signalling and could potentially be used as therapeutic agents to promote regeneration of the mammalian spinal cord and central nervous system. Differential gene profiling of spinal cords constitutively expressing RARβ2 have identified chemokine signalling pathways components, CCL5 and CXCR4, whose expression was downregulated 100 and 7 fold respectively, compared to normal spinal cords. Normally, in the injured mammalian spinal cord, CXCR4 expression is upregulated. The reasons for the differences expression of CXCR4 between regenerating and non-regenerating animals are not yet full known. Since CXCR4 signalling has been shown to be a key factor in axonal guidance during development, and a necessary component of epimorphic regeneration in certain animals, it is likely that CXCR4 signalling would be necessary for spinal cord and tail regeneration in the adult newt.

2.13 Final Comments

The involvement of CXCL12/CXCR4 signalling during the regeneration of the central nervous has been mainly limited to studies involving the migration of neural stem cells and neural precursor cells. The possible roles of CXCL12/CXCR4 signalling during axon guidance, cell recruitment, and redifferentiation in the regenerating spinal

cord of the newt have yet to be investigated. Also, evidence has not yet been provided regarding the role of chemokine signalling through CXCR4 as a downstream target of retinoid signalling in the regenerating urodele spinal cord.

Chapter 3: Materials and Methods

3.01 Tail Amputation and Blastema Removal

Adult newts, *Notophthalmus viridescens* were purchased from Boreal Labs (St. Catharines ON). All protocols were approved by the Brock University Animal Care and Use Committee. The newts were anaesthetized by submersion in 0.1% MS222 (Sigma-Aldrich) at pH 7.0. The skin was sterilized by immersion (15 s) in 0.1 % chloramine T (Sigma-Aldrich) and rinsed in sterile amphibian's Ringer's solution. The tail was amputated approx. 2cm distal from the cloaca using a sterile razor blade. All equipment was sterilized using 70% ethanol. Tail blastemas were removed using similar procedures at 0, 2, 4, 6, 8, 12, and 21 days post amputation. Blastemas were briefly placed in chloramine T, rinsed in sterile amphibian Ringer's solution containing antibiotic and antimycotic (Ringer's + AA) (GIBCO). Next, blastemas were flash frozen in liquid nitrogen and stored at -80° C for future use.

3.02 Spinal Cord Removal and Culture

Once the newts were anaesthetized and sterilized, they were decapitated and the vertebral column was dissected out of the body. The spinal column was sterilized by immersion in 0.1% chloramine T, and rinsed in sterile amphibian Ringer's + AA. The spinal cord was carefully dissected out of the spinal column and placed in amphibian Ringer's + AA for 10 – 15 min while the meninges were removed with fine forceps. The spinal cord was then cut into 0.5 mm transverse sections using a scalpel. The spinal cord sections were then dipped in chloramine T, rinsed in sterile Ringer's + AA and then rinsed in L-15 defined medium. Spinal cord sections were promptly cultured.

3.03 Culture conditions

Spinal cord explants were cultured on poly-L- Lysine (Sigma-Aldrich) coated glass coverslips (Bellco) in 35 mm culture dishes (Fisher). No more than three spinal cord explants were cultured in each dish. To aid attachment of the explants to the dish, the explants were cultured for 10-20 minutes in a minimal volume of L-15 defined medium. After attachment, the volume of medium was adjusted to 3.0 ml. Spinal cord explants were cultured for 72 hours at 21°C in the dark.

Spinal cord explants were cultured in 70% Leibovitz L-15 medium (GIBCO). The defined medium (DM) was supplemented with 1% glucose, 300 ng/ml glutamine, 125 U/ml penicillin, 125 µg/ml streptomycin, 0.14U/ml insulin (all GIBCO), 6.3 ng/ml progesterone (Sigma-Aldrich), and 5 µg/ml transferrin (Sigma-Aldrich). The pH of the culture medium was adjusted to 7.5-7.6. All-trans-RA (Sigma-Aldrich) stocks (10^{-2}M) were prepared fresh in 100% ethanol in low light conditions and diluted to a final concentration of 1×10^{-7} M in DM. The synthetic CXCR4 antagonist, AMD3100 (Sigma-Aldrich) was dissolved in phosphate buffered saline (PBS). AMD3100 stocks (1×10^{-2} M) were aliquoted and stored at -20°C and diluted to a final concentration of 1×10^{-5} M with medium just prior to use. CXCL12 (R&D Systems) was reconstituted in PBS and stock concentrations (100 µg/ml) were stored at -80°C in aliquots to avoid freeze thaw cycles. In the absence of all-trans RA, AMD3100 or CXCL12, vehicle (ethanol (EtOH) or PBS) concentrations were adjusted to match treatments containing the appropriate agents.

3.04 Analysis of Axon Outgrowth

Spinal cord explants were examined for axonal outgrowth (also known as neurite extension) 72 hours after being cultured. The cultured explants were viewed using a Zeiss Axiovert 200 inverted microscope and imaged using a digital camera and Northern Eclipse (Empix Imaging, ON) software. Individual axons were traced and measured digitally, from the perimeter of the explant to the visible tip of the growing axon.

For axonal length, the data were analyzed using a Kruskal-Wallis One Way Analysis of Variance on Ranks, and the Bonferroni corrected (step down) t-tests, and considered significant with $p < 0.05$.

3.05 Cloning of CXCR4 cDNA

A partial cDNA fragment was previously cloned in our laboratory by Christopher Carter and sequenced at The McGill University and Genome Québec Innovation Centre (Fig. A1).

3.06 5' and 3' RACE

Total RNA was isolated as previously outlined. Brain tissue was used because initial results indicated that it contains higher levels of CXCR4 mRNA. Amplification of 5' and 3' cDNA ends was done according to the manufacturer's instructions (SMART RACE cDNA Amplification Kit, Clontech). Primers were designed from the partial CXCR4 sequence. CXCR4 gene specific race primers and nested RACE primers were as follows:

5' end RACE primer 5' GGTTTGTTCTGCCTGGCCTGGTCATC 3',

3' end RACE primer 5' TATGAAGTCGGGCACGGTCAAGAGCA 3'.

5' end NESTED RACE primer 5' TCTAGGCTGTCCCACTCCAAAGGCCA 3',

3' end NESTED RACE primer, 5' GTTGGTGGCGTGGACTATCGCCAAGT 3'.

Nested RACE products were purified using the QIAquick Gel Extraction Kit (QIAGEN), and ligated into the pGEM –T Easy vector (Promega) and then transformed into JM109 competent *E.coli* (Promega) using heat shock. Transformed JM109 cell cultures were plated onto LB ampicillin plates supplemented with X-gal for blue/white screening as outlined in Sambrook and Russel (2001).

White colonies were picked and grown overnight in LB broth with ampicillin. Plasmids were then isolated using the QIAprep Spin Miniprep Kit (QAIGEN). Isolated plasmids containing RACE products were then sent for sequencing to The McGill University and Genome Québec Innovation Centre.

3.07 Reverse Transcription PCR

Regenerating tail blastemas were collected as previously described. The working areas and all equipment were cleaned with RNA Zap (Sigma). RNase free plasticware was used in the RNA isolation and handling process. Total RNA was isolated using TRIZOL (Invitrogen) following the manufacturers protocol. RNA samples were treated with Turbo DNase (Ambion). RNA concentrations were measured using an Ultrospec 2100 pro spectrophotometer at 260 nm. RNA samples were stored at -80°C, and kept on ice during handling. Reverse transcription (RT) was carried out using the RETROscript kit

(Ambion). Depending upon the amount of tissue available for total RNA isolation, RNA concentrations of 2.0 µg or 0.75 µg of total RNA were used for RT-PRC analysis. The amount of total RNA (2.0 µg or 0.75) was kept constant within each set of RT-PRC reactions. PCR was done using SuperTAQ Polymerase (Ambion). A total of 29 cycles were used for amplification at annealing temperatures of 62°C (5 cycles) and 68°C (25 cycles). The primer sequences of CXCR4 and all control genes, elongation factor 1 alpha (EF1α), beta actin, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are as follows:

CXCR4 forward 5'CTCTACAGCAGCGTCYTGATC 3',

CXCR4 reverse 5' AGGCAGATGTARTARGGCAAC 3'.

EEF1α forward 5' CCATGTGTGTGGAGGAGAGCTTCTCA 3',

EF1α reverse 5' GGCTCTTGTGATGGACCCTAATC 3',

β *Actin* forward 5' CCACTGCTGCTTCTTCATCCTCTC 3',

β *Actin* reverse 5' GGCACCTGAACCGCTCATTG 3',

GAPDH forward 5' TGTGGCGTGACGGCAGA 3',

GAPDH reverse 5' AAGCGGCAGGTCAGGTCAA 3'.

Products were analyzed by agarose gel electrophoresis (1% agarose) after staining with GelRed (Biotium). By diving the band intensity of CXCR4 to the relative control, the expression level is shows as a percent.

3.08 *In Vivo* Bead Implantations

AG-1X2 beads (BioRad) were washed at least 5 times (10 min intervals) in 1X PBS and soaked in AMD3100 (1X 10^{-3} M) for 72 hours in the dark at 4°C. RA beads were soaked in RA (4mg/ml) dissolved in 100% ethanol and stored at -20°C for 72 hours. LE135 beads were soaked in LE135 (2 X 10^{-3}M) dissolved in DMSO and also soaked in DMSO for 72 hours at 4°C. The LE135 antagonist has been shown to bind to RARβ (Eyrolles et al., 1994; Kagechia, 2002), and was a generous gift from Dr Kagechia (University of Tokyo). AMD3100 has been shown to bind to and inhibit CXCR4 signalling (Toba et al., 2007). All beads were briefly rinsed twice in sterile amphibian Ringers solution before implantation. Control beads were also soaked for 72 hours in their corresponding solvents (PBS, EtOH, and DMSO) under the appropriate conditions.

For implantation, the newts were anesthetised and the tail was amputated as previously described. Beads were implanted at three and six days post amputation. Using fine forceps, a small hole was made through the wound epithelium in the regenerating tail blastema. The beads were carefully placed in the regenerating tail blastema. Three beads were implanted for each treatment. The tails were allowed to regenerate for 72 hours after implantation until blastemas were harvested for analysis

3.09 Histology

Tail blastemas containing bead implants were collected from anesthetised animals as previously described. Basic histological techniques were adapted from

Humason (1962). Blastemas were promptly fixed in 10% formalin. After fixation, blastemas were rinsed and decalcified in 10% formic acid for 3 hours and then rinsed in running tap water for 15 min. Tissues were then dehydrated using increasing concentrations of ethanol, (50%, 70%, 95%, and 100% x2) for 15 minutes each and then cleared in 100% methyl salicylate. Once cleared, blastemas were infiltrated with PARAPLAST tissue embedding medium (Fisher) and embedded. Tissue sections were cut 14μm thick and attached to Superfrost microscope slides (Fisher).

The slides were deparaffinised in xylene and hydrated to distilled water using decreasing ethanol bath concentrations (100%, 95%, 70%, 50%, distilled water) for 3 minutes each and finally 3% glacial acetic acid for 3 minutes. Next, slides were stained in 1% Alcian blue (Sigma-Aldrich)(in 3% glacial acetic, pH 2.5) for 30 minutes. Slides were washed in running tap water for 3 minutes and then rinsed in distilled water. Sections were then counterstained using Nuclear Fast Red 0.1% (Sigma) for 5 minutes, washed in running tap water and then dehydrated in a series of increasing ethanol bath concentrations (3 min each) and two changes in 100% ethanol. Next, the sections were briefly cleared in xylene and mounted in a resinous mounting medium (DPX mountant, BDH Chemicals).

Photomicrographs were made using a Zeiss compound light microscope and a Nikon Eclipse E200 digital camera using NIS Elements F 3.0 software

Chapter 4: Results

4.01 Isolation of CXCR4 Clone Using 5' and 3' RACE

The expression of CXCR4 in the central nervous system of adult *N. viridescens* has previously been confirmed, and a cDNA fragment was provided courtesy of Chris Carter from our laboratory (Figure A1). A full length cDNA sequence of CXCR4 would provide valuable information regarding amino acid composition and homologies with other species, all of which would be useful for the investigation of its expression pattern and role during spinal cord and tail regeneration in *N. viridescens*. With a partial sequence already known, we used the 5' and 3' RACE (Rapid Amplification of cDNA Ends) technique to determine the full length coding sequence of CXCR4.

Fresh, total RNA was isolated from newt brain tissue (previously described in Materials and Methods) to prepare RACE ready cDNA. Initial results from 5' and 3' RACE reactions did not yield distinct RACE products. The next step was to use the Nested RACE technique, which did produce distinct RACE products from the 5' and 3' RACE reactions. A single band of approximately 500 base pairs (bp) was produced from the Nested 5' RACE reaction, and the 3' Nested RACE reaction produced a single band with an extremely strong signal of approximately 800 bp in size (Figure 1). The RACE products were then excised from the agarose gel, ligated into the pGEM-T Easy vector, transformed into JM109 competent cells and grown on a selective medium. Plasmids were isolated and the inserts sequenced (previously described in Materials and Methods).

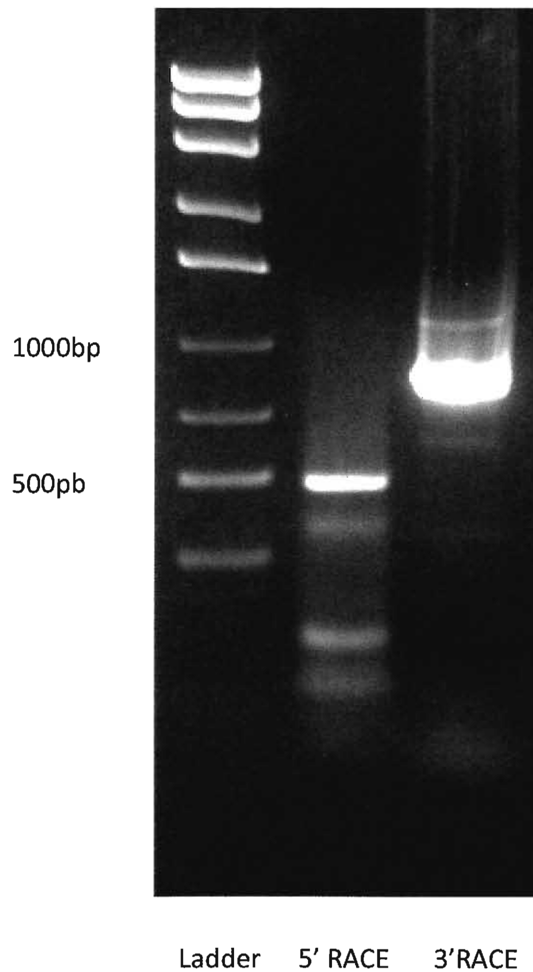

Figure 1. 5’ and 3’ Nested RACE products from CXCR4 cDNA template. Using RACE products from initial RACE results, the Nested RACE technique produced distinct 5’ products (approx. 480 bp) and 3’ products (approx. 800 pb). The smaller, less distinct bands seen in the 5’ and 3’RACE reactions are incomplete RACE products.

Sequencing results from 5′ and 3′ Nested RACE products were confirmed by BLAST analysis. The 5′ and 3′ cDNA sequences were then pieced together with the original partial sequence by using the overlapping regions from the RACE products and the known partial sequence (Figure 2). BLAST results of the new CXCR4 cDNA sequence further confirmed our initial results and suggested that the full length cDNA had been sequenced (Figure A2). Multiple alignment comparisons of newt CXCR4 show distinct regions of conserved homologies. *N. viridescens* CXCR4 showed 73%, 76%, and 77% amino acid homology with *Xenopus Laevis* (African Clawed Frog), *Oryctolagus cuniculus* (European Rabbit) and *Homo sapiens* (Humans) respectively. There are regions in the amino acid sequence which do not seem to be conserved across species, particularly at the N-terminus. Other regions that have low homology include the extracellular domains (represented by boxed regions in Figure 3). These extracellular domains, which are likely involved in ligand binding, appear to have the lowest level of sequence homology between species (Figure 3). Thus the full length sequence has not been conclusively determined, but our knowledge of the sequence has been significantly expanded.

A

5’GAGGATTCATCATGGATAACATGGATATTTCAGGTGAACTCATCATCAACATGCCCAATGATTACACGG
ATAACACGACAGATGGCATGGCTTCGGGGGACATGGATTACCTGGAGGAGCCGTGCTTCAAGAATAACA
CTTTCAACCGGATATTCTTGCCAACGATGTACTCCATCATCTTCCTAATGGGAATCATTGGCAACGGGTTG
GTTGTCCTTGTCATGGGCTACCAGAAGAAATCAAGGACTATGACGGACAAGTACCGGCTGCACCTGTCTG
TGGCCGATCTTCTGTTTGTCTTCACCTTGCCCTTCTGGTCCGTAGATGCTGTGATAGGCTGGTGTCTTAAA
GAGTTCCTCTGCAAAGCCGTTCACGTCATCTACACAGTCAACCTCTACAGCAGCGTCCTGATCTTAGCCTT
CATAAGTTTAGACCGCTACTTGGCGATAGTCCACGCCACCAACAGCCAAGGTACGAGGAAGCTGCTGGC
TGAGAAGGTGGTGTATGCTGGGGTGTGGTTGCCGGCTGTGCTCTTGACCGTGCCCGACTTCATATTTGCC
AGTGTTAGTATGGAAGAAGGCACCTACGAATGCAACCACATCTATCCCTACGAGACGCGGAAAGAGTGG
ACTGTTGGATTTAGGTTTGTGCACCTCACGGTAGGGTTTGTTCTGCCTGGCCTGGTCATCTTGATTTGCTA
CTGCATCATAATTTCTAGGCTGTCCCACTCCAAAGGCCACCAGAAGCGCAAAGCATTGAAGACGACGGTG
ATTCTCATCCTCACCTTCTTCACCTGCTGGTTGCCCTACTACATCGCCCTCAGCATAGACACCTTCATACAA
ATGGGCGTGATCAAGAACATTACATGTGACTTTGAGGGCACTCTGGGTACGTGGATCTCCATCACTGAAG
CCTTGGCCTTCTTCCACTGCTGCCTGAACCCCATCCTCTACGCCTTCCTCGGGGCCAAATTCAAGAGCACC
GCTCAGAATGCCCTCACCACGGTGAGCAGAGGATCCAGCCTGAAGATACTGTCCAAAAAGCGAGCAGGG
CACTCGTCAGTTTCGACAGAGTCTGAATCGTCGAGTTTCCACTCTAGTTAACGCAGAGTCGCGCCTCATG
GGACTGTGTTACTTTTGGACTGCACGCCTCCGAGTGACCCCCGCGTACTCTGCGTTGATACCACTG 3’

B

MDNMDISGELIINMPNDYTDNTTDGMASGDMDYLEEPCFKNNTFNRIFLPTMYSIIFLMGIIGNGLVVLVMG
YQKKSRTMTDKYRLHLSVADLLFVFTLPFWSVDAVIGWCLKEFLCKAVHVIYTVNLYSSVLILAFISLDRYLAIVH
ATNSQGTRKLLAEKVVYAGVWLPAVLLTVPDFIFASVSMEEGTYECNHIYPYETRKEWTVGFRFVHLTVGFVL
PGLVILICYCIIISRLSHSKGHQKRKALKTTVILILTFFTCWLPYYIALSIDTFIQMGVIKNITCDFEGTLGTWISITEAL
AFFHCCLNPILYAFLGAKFKSTAQNALTTVSRGSSLKILSKKRAGHSSVSTESESSSFHSS

Figure 2. Full length coding sequence of *Notophthalmus viridescens* CXCR4.

(A) 5’ to 3’ cDNA sequence of *N. viridescens* CXCR4

(B) Deduced amino acid sequence of *N. viridescens* CXCR4. Original partial sequence is highlighted

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
N. viridescens  MDNMDISGEL IINMPNDYTD NTTDGMASGD MDYLEEPCFK --NNTFNRIF
X. laevis       MD--GFSGGI DINI---FDG NSTE-NGSGD FEDFIEPCFM QENSDFNRIF
O. cuniculus    ---------- -------TSD NYTEELGSGD YDSIKEPCFR EENAHFNRIF
H. sapien       ME--GISSIP LPLLQIYTSD NYTEEMGSGD YDSMKEPCFR EENANFNKIF

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
N. viridescens  LPTMYSIIFL MGIIGNGLVV LVMGYQKKSR TMTDKYRLHL SVADLLFVFT
X. laevis       LPTIYSFIFL LGIIGNGLVV VVMGYQKKSR TMTDKYRLHL SVADLLFVFT
O. cuniculus    LPTIYSIIFL TGIVGNGLVI LVMGYQKKQR SMTDKYRLHL SVADLLFVIT
H. sapien       LPTIYSIIFL TGIVGNGLVI LVMGYQKKLR SMTDKYRLHL SVADLLFVIT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
N. viridescens  LPFWSVDAVI GWCLKEFLCK AVHVIYTVNL YSSVLILAFI SLDRYLAIVH
X. laevis       LPFWSVDAAI GWYFKEFLCK AVHVIYTVNL YSSVLILAFI SLDRYLAIVH
O. cuniculus    LPFWAVDAVA NWYFGKFLCK AVHVIYTVNL YSSVLILAFI SLDRYLAIVH
H. sapien       LPFWAVDAVA NWYFGNFLCK AVHVIYTVNL YSSVLILAFI SLDRYLAIVH

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
N. viridescens  ATNSQGTRKL LAEKVVYAGV WLPAVLLTVP DFIFASVSME EGTYECNHIY
X. laevis       ATNSQGSRKM LADKVVYAGV WLPALLLTVP DLVFASVSNE NGQFVCDRIY
O. cuniculus    ATNSQKPRKL LAEKVVYVGV WIPALLLTIP DFIFANVREA EGRYICDRFY
H. sapien       ATNSQRPRKL LAEKVVYVGV WIPALLLTIP DFIFANVSEA DDRYICDRFY

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
N. viridescens  PYETRKEWTV GFRFVHLTVG FVLPGLVILI CYCIIISRLS HSKGHQKRKA
X. laevis       PIDNRETWTV GFRFLHITVG LILPGLIILV CYCVIISKLS HSKGHQKRKA
O. cuniculus    PSD---LWVV VFQFQHIMVG LILPGIVILS CYCIIISKLS HSKGHQKRKA
H. sapien       PND---LWVV VFQFQHIMVG LILPGIVILS CYCIIISKLS HSKGHQKRKA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
N. viridescens  LKTTVILILT FFTCWLPYYI ALSIDTFIQM GVIRNITCDF EGTLGTWISI
X. laevis       LKTTVILILA FFACWLPYYV CLTTDTFMML GLVR-ADCIW ENTLHKAISI
O. cuniculus    LKTTVILILA FFACWLPYYI GISIDSFILL EIIR-QGCEF ENTVHKWISI
H. sapien       LKTTVILILA FFACWLPYYI GISIDSFILL EIIR-QGCEF ENTVHKWISI

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
N. viridescens  TEALAFFHCC LNPILYAFLG AKFKSTAQNA LTTVSRGSSL KILSK-KRAG
X. laevis       TEALAFFHCC LNPILYAFLG AKFKRSAQNA FTSVSRGSSL KILSK-KRAG
O. cuniculus    TEALAFFHCC LNPILYAFLG AKFKTSAQHA LTSVSRGSSL KILSKGKRGG
H. sapien       TEALAFFHCC LNPILYAFLG AKFKTSAQHA LTSVSRGSSL KILSKGKRGG

                ....|....| ....|.
                       360
N. viridescens  HSSVSTESES SSFHSS
X. laevis       LSSVSTESES SSFHSS
O. cuniculus    HSSVSTESES ------
H. sapien       HSSVSTESES SSFHSS
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Figure 3. Multiple alignment of CXCR4 amino acid sequences. Amino acid sequence sharing homologies of ≥75% are highlighted. Gaps are indicated by (-). Boxed regions represent extracellular domains. *X. laevis* NCBI Reference Sequence: ref NP_001080681.1, *O. cuniculus* GenBank: ABX55952.1, *H. Sapiens* GenBank: CAA12166.1

4.02 CXCR4 is Expressed in all Tissues Examined in the Adult Newt

The next objective of this study was to determine where CXCR4 was expressed in *N. viridescens*. Various tissues (brain, spinal cord, heart, gut, liver) were used for this study. Central nervous system tissues (brain and spinal cord) were chosen because it is the system of focus in this study. Heart, gut and liver were chosen because of abundance and ease in obtaining enough tissue and their regenerative abilities within this species. Total RNA was isolated (previously described) from pooled tissue samples from at least 3 newts. Using reverse transcription PCR (RT-PCR) we were able to detect CXCR4 expression in these specific tissues and compare its relative expression levels (normalized against constitutively expressed controls) among these tissues. Results indicated that CXCR4 expression is detectable in all tested tissues (Figure 4). The highest level of expression was in the brain, followed by the liver. The spinal cord, heart, and gut display lower levels of expression. It is not surprising to see such diverse expression of CXCR4, because chemokine signalling is involved in a wide variety of processes, including those of the immune system (Li and Ransohoff, 2007).

In the brain, gut and liver tissue, RT-PCR resulted in a smaller, less defined band visible just below the band representing CXCR4. The presence of this signal suggests that there may be more than one isoform of CXCR4 being expressed in the newt. This is a very likely possibility since multiple isoforms of CXCR4 have been confirmed in other organisms. For example, Dufourcq and Vrix (2006) demonstrated that CXCR4a and CXCR4b are expressed during zebrafish fin regeneration. These results show that CXCR4

is widely expressed in *N. viridescens*, and that the presence of 2 isoforms is possible, but is yet to be confirmed in the newt.

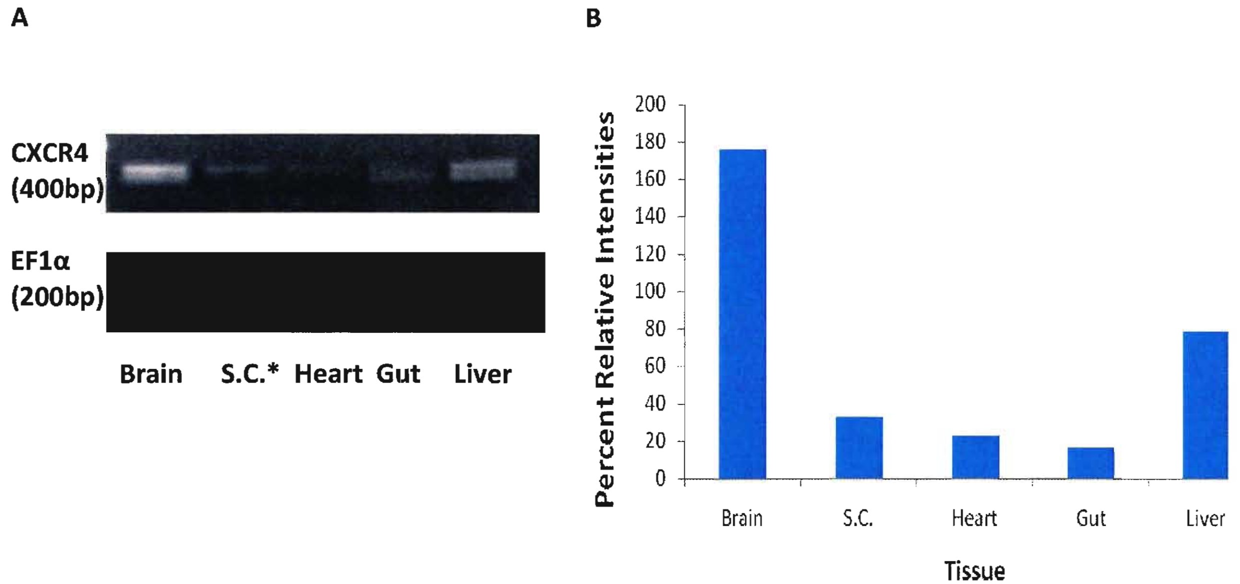


Figure 4. Newt CXCR4 is expressed in all regenerative tissues tested.

(A) CXCR4 was detected in all tested tissues. Brain tissue had the highest relative expression of CXCR4 under normal conditions. EF1α was used as control probe to compare relative levels of gene expression to CXCR4. 20μl of each PCR product was loaded into each well. S.C. is spinal cord. The experiment was repeated twice using fresh RNA from new tissue samples (n=2).

(B) Percent relative band intensities of CXCR4 to EF1α at various time points of regeneration. ImageJ 1.42q software was used for densitometric gel analysis.

4.03 CXCR4 is Upregulated in Tail Blastemas During the Early Stages of Tail Regeneration

Upon confirming the expression of CXCR4 in *N. viridescens,* the next step was to elucidate the expression pattern of CXCR4 during the process of tail regeneration. Newt tails were amputated about 1/3 the distance distal from the cloaca (approx. 2 cm) and allowed to regenerate. Blastemas were collected at various time points after amputation by re-amputation of the tail approximately 2-3mm proximal to the original amputation site. Tissue samples were pooled (3 blastemas for each day of regeneration). RT-PCR was performed as previously described. RT-PCR results show an upregulation of CXCR4 from day 0 up to day 12 of regeneration (Figure 5). Compared to Day 0, an increase of 26% is seen on day 2 of regeneration. From here, the rate of increase of CXCR4 expression plateaus and does not begin to increase until days 6 and 8. Day 12 showed an expression level 80% above that of day 0, the highest expression level of CXCR4 from any of the days tested. By day 21, CXCR4 expression has returned to basal levels. Normalizing controls *EF1α*, *GAPDH*, and *actin* did not display any changes in their expression levels during the same time of regeneration (Figure 5). These results suggested that during the early stages of tail regeneration in *N. viridescens*, CXCR4 is upregulated at the mRNA level of gene expression.

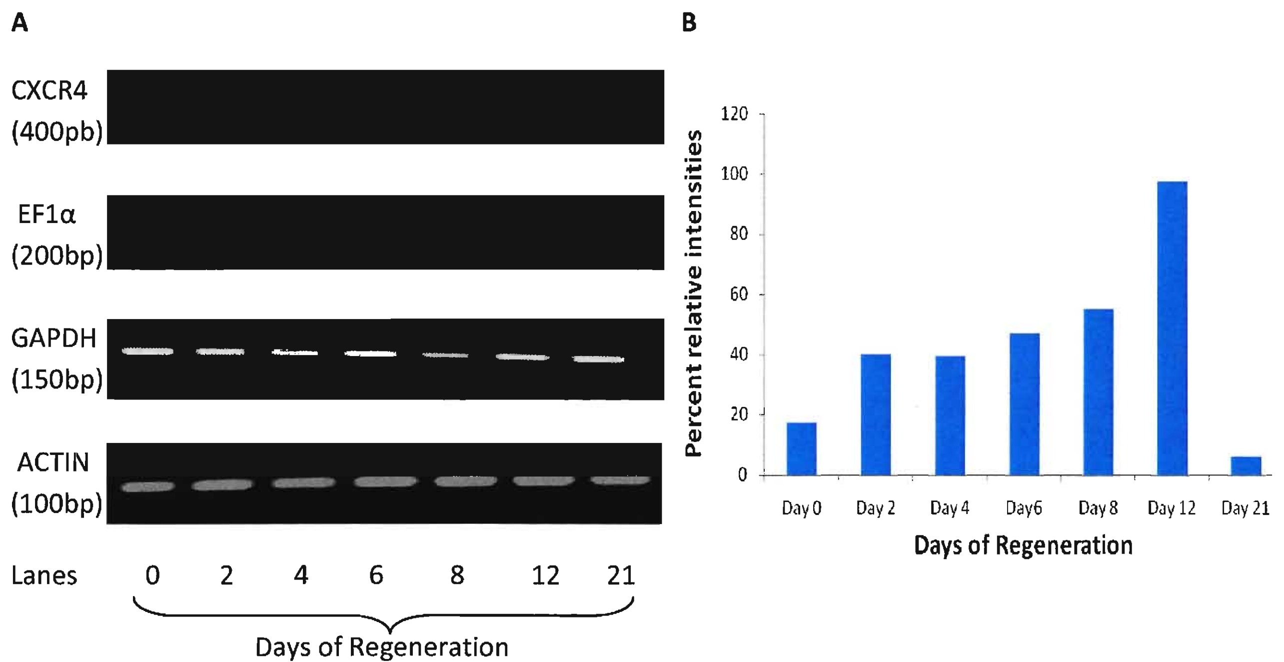


Figure 5. Newt CXCR4 expression is increased during the early post-amputation stages of tail regeneration.

(A) Total RNA was isolated from regenerating newt tail blastemas at the indicated days post amputation. RT-PCR demonstrated a steady increase in CXCR4 expression from day 0 up to day 12. CXCR4 returned to basal expression levels by day 21 of regeneration. *EF1α*, *GAPDH*, and *Actin* were used as controls to compare relative levels of gene expression to CXCR4. Each well was loaded with 20μl of PCR product. The experiment was repeated twice using fresh RNA from new tissue samples (n=2).

(B) Percent relative band intensities of CXCR4 to *Actin* at various time points of regeneration. ImageJ 1.42q software was used for densitometric gel analysis.

4.04 *In vivo* AMD3100 Inhibition of CXCR4 Signalling Differentially Regulates the Expression of CXCR4 in Tail Regenerates at Day 3 and Day 6 Post Amputation

The next objective was to gather information regarding the function of CXCR4 during tail regeneration. We approached this by using a bead implantation technique that releases a CXCR4 inhibitor directly into the regeneration tail blastema that should diffuse into the surrounding tissues at the early stages of regeneration (between day 3 and 12 of regeneration). We hypothesized that this would temporarily reduce or abolish CXCR4 signalling during this critical period of regeneration. We used the CXCR4 specific inhibitor, AMD3100, which has previously been used to study chemokine signalling in the rat CNS (Lazarini et al., 2000). If CXCR4 signalling is necessary for normal regeneration to occur, we might expect to see changes in the level of expression of CXCR4 and possible gross morphological tissue/cellular effects in the regenerating tail if CXCR4 signalling has been reduced or abolished. Perturbations in signalling pathways, such as the early misexpression of CXCL12 have been shown to inhibit the pathway and shut down or prevent regeneration in the zebrafish fin (Dufourcq and Vriz, 2006). Chemical inhibition of CXCR4 signalling with AMD3100 has also been shown to reduce CXCR4 transcript adundance (Hu et al., 2006).

Due to the nature of the bead implant study, where the beads release the inhibitor over time, the exact concentration, rate of release, and diffusion of the inhibitor is unknown. Thus we implanted the beads at day 3 and day 6 of regeneration to determine which stages, if any, were susceptible to the inhibitory effects of AMD3100

and had the greatest effect on CXCR4 expression, and subsequent signalling. These time points were chosen because CXCR4 expression was shown to sharply increase between day 2 and 12 of regeneration, suggesting a greater importance of CXCR4 signalling during this time period of regeneration. All tail blastemas with bead implants were collected for analysis at day 14 of regeneration.

AMD3100 beads implanted at day 3 post-amputation resulted in an approximate 17% increase in CXCR4 expression at the mRNA level from control implants (Figure 6). Interestingly, day 6 AMD3100 bead implants had the opposite effect on CXCR4 expression, resulting in an approximate 45% decrease in CXCR4 mRNA expression compared to controls (Figure 6). These changes in the relative expression levels of CXCR4 suggest that CXCR4 signalling may be more sensitive to inhibition by AMD3100 after day 6 of regeneration, when CXCR4 is approaching its highest measured expression levels.

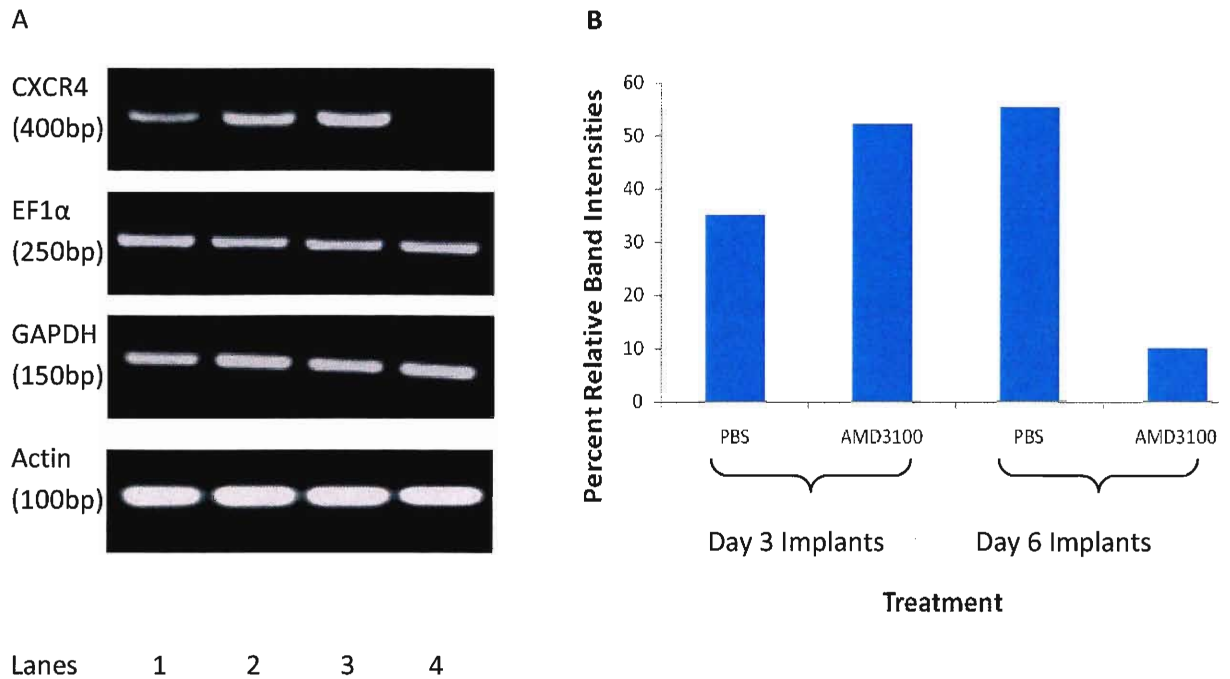


Figure 6. CXCR4 expression is differentially regulated by AMD3100 at day 3 and day 6 of post amputation

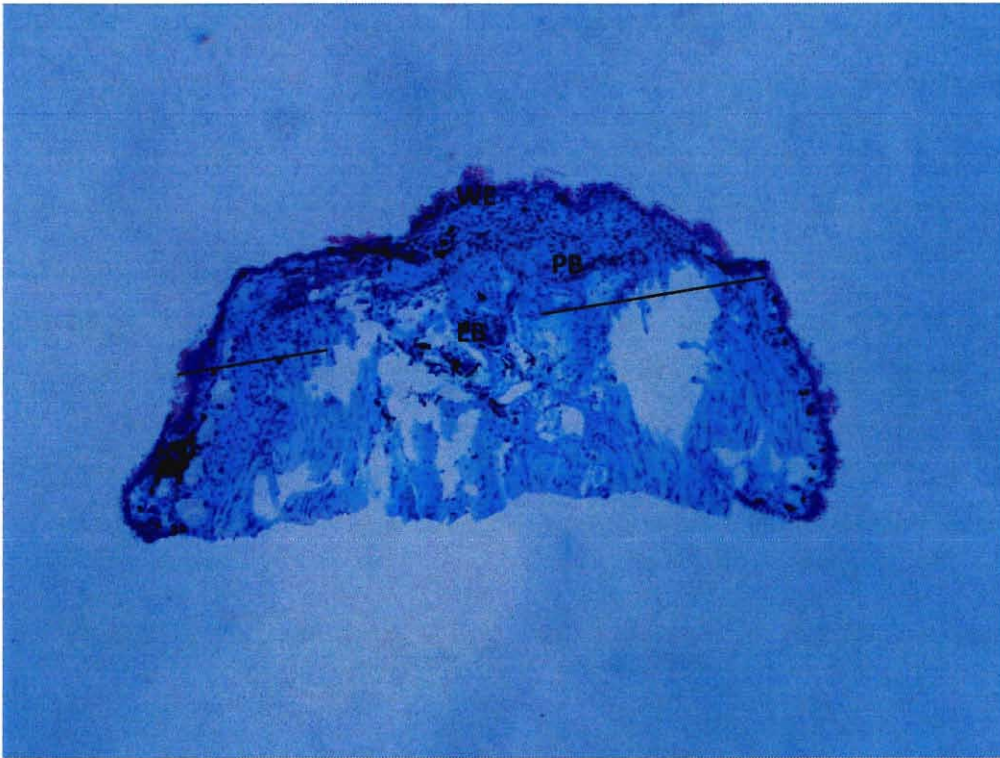
(A)Beads were treated with AMD3100 or PBS (control) and implanted at day 3 or day 6 of tail regeneration. All implanted tail blastemas were collected at day 14 of regeneration and total RNA was isolated and analyzed for CXCR4 expression. Lane 1; Day 3 PBS implant, Lane 2; Day 3 AMD3100 implant, Lane 3; Day 6 PBS implant, Lane 4; Day 6 AMD3100 implant. 20µl of each PCR product was loaded into each well. n=1.

(B) Percent relative band intensities of CXCR4 to Actin for all treatment conditions

4.05 Inhibition of CXCR4 Signalling by AMD3100 at 6 Days Post Amputation Inhibits Ependymal Outgrowth

The control tail regenerates (PBS beads implanted at day 6) showed typical histological features of a regenerating tail. The AMD3100 treated tail has a much thicker wound epithelium than the control tail blastema (Figure 7). Degenerating muscle fibres, which are normally seen during the process of tissue reorganization in early regeneration, were only clearly seen in the control tail sections. The ependymal tube and terminal vesicle projecting into the regenerating tail blastema can be seen in figure B. In figure A, only what appears to be the ependymal bulb can be seen at the distal end of the spinal cord that has receded into the tail stump and has not regenerated. Unfortunately, the small sample size of this experiment (n=1), cannot allow us to draw many solid conclusions. This preliminary result does support other experimental results and our hypothesis that functional signalling of CXCR4 is critical for normal regeneration to take place.

A



B

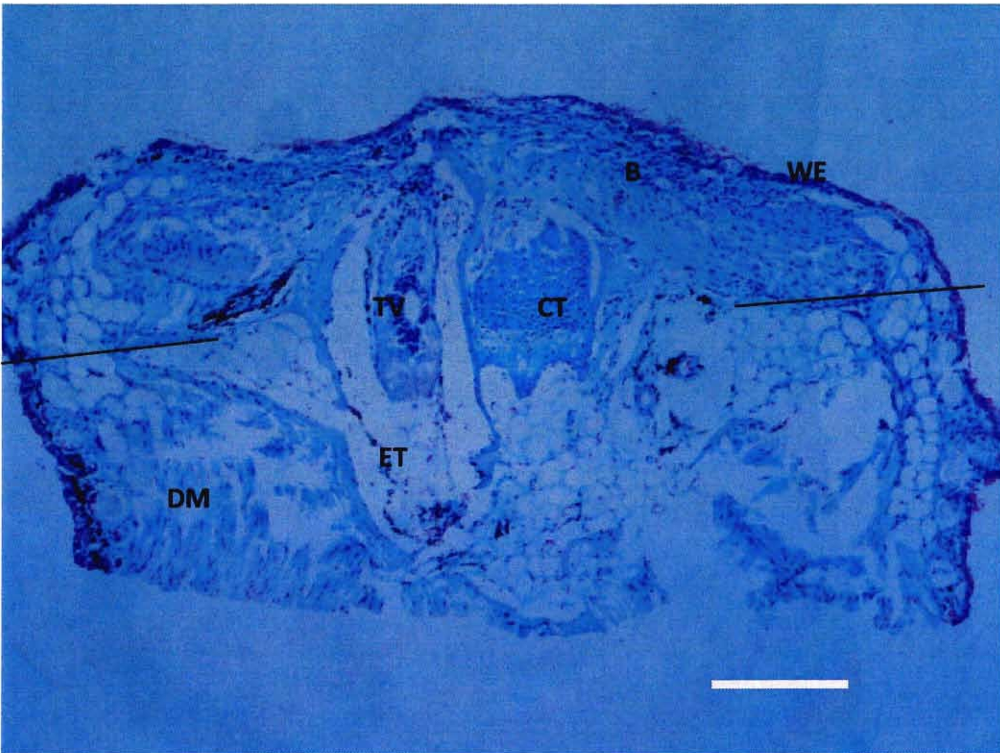


Figure 7. Inhibition of CXCR4 signalling inhibits spinal cord regeneration

Midsagittal section of day 12 regenerated tail blastemas implanted with AG1X2 beads at day 6 post amputation. Regenerating newt tails were implanted with three to four AG1X2 beads soaked in various agents. Tail blastemas were then allowed to regenerate to day 14 of regeneration. Representative midsagittal sections of a regenerating tail blastema are shown. (40X magnification). The initial plane of amputation is shown by the solid lines. Wound epithelium (WE) , blastema (B), pseudoblastema (PB) , terminal vesicle (TV), ependymal tube (ET), ependymal bulb (EB), degenerating muscle (DM), and connective tissue (CT) are indicated in the figure. Scale bar = 1 mm

(A) Regenerating tail blastema implanted with AG1X2 beads soaked in AMD3100 (CXCR4 inhibitor) at day 6 of regeneration.

(B) Regenerating tail blastema implanted with AG1X2 beads soaked in PBS (vehicle control) at day 6 of regeneration.

4.06 Both atRA and AMD3100 Downregulate CXCR4 Expression *in vivo*

Evidence gathered thus far suggests that the regenerating tail is sensitive to changes in chemokine signalling during the early stages of tail regeneration. The next objective of this study was to determine if retinoid signalling is somehow linked to chemokine signalling during regeneration.

Three separate bead implantation studies were performed. All beads were prepared as described in the methods. In the first experiment AMD3100 soaked beads were implanted to again assess the effects of inhibition of CXCR4 signalling on CXCR4 mRNA expression. Next we wanted to determine if the addition of beads treated with all trans retinoic acid (atRA) would affect the level of CXCR4 expression. Finally we assessed if the expression of CXCR4 would be altered by inhibiting atRA/RARβ signalling with the use of LE135, an RARβ specific antagonist.

All beads were implanted at day 6 of regeneration, and blastemas were collected 72 hours after implantation. Results from RT-PCR analysis of CXCR4 expression are shown in Figure 8. Once again, treatment with AMD3100 resulted in the downregulation of CXCR4 mRNA levels relative to controls. Interestingly, treatment with atRA also resulted in the down regulation of CXCR4. We also found that LE1235 caused a slight increase in CXCR4 expression, but the effect of the DMSO (vehicle control) on CXCR4 expression is very large and prevented us from clearly observing if LE135 did effect CXCR4 expression. These results are consistent with our earlier findings that CXCR4 is downregulated at day 6 of regeneration by AMD3100 and that RA signalling,

possibly through RARβ, can also down regulate expression of CXCR4. These results suggest a functional link between these two signalling pathways.

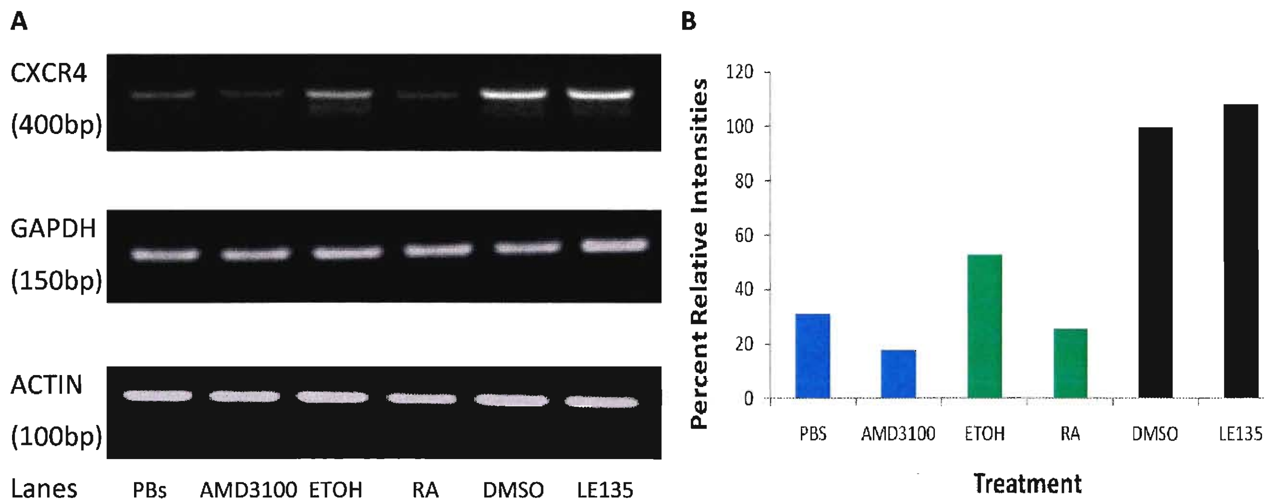


Figure 8. RT-PCR of total RNA from AG1X2 bead implanted at day 6 post amputation

(A) AG1X2 resin beads were soaked in AMD3100, LE135, RA, and their respective vehicle controls, PBS, DMSO, or EtOH, all for 72 hours and implanted into the regenerating newt tail blastema at day 6 of regeneration. Tail blastemas for RT-PCR were collected 72 hours after implantation. 20µl of each PCR product was loaded into each well. n=1.

(B) Percent relative band intensities of CXCR4 to Actin for all treatment conditions. ImageJ 1.42q software was used for gel analysis.

4.07 Altered Chemokine and Retinoid Signalling Directly Affect Neurite Outgrowth in Spinal Cord Explants of *N. viridescens*

Our results show that perturbations to CXCR4 signalling can be detrimental to tail regeneration and also spinal cord regeneration. Previous studies have shown that treating newt spinal cord explants with various factors under specific culture conditions can produce enhanced neuronal outgrowth (Dmetrichuk, 2005). Two indicators of *in vitro* spinal cord regeneration were measured: the number of neurites growing from the explant, and mean neurite length.

Statistical analysis of the three control groups (PBS, EtOH, PBS+EtOH) showed no significant difference ($p < 0.05$). In an attempt to simplify our analysis, we pooled all control data into one treatment group.

Mean number of neurite outgrowths from RA, CXCL12, and RA+CXCL12 treatments were significantly different from controls (Figure 9). Treatment with AMD3100 had no significant effect on its own ($p > 0.05$). However, explants treated simultaneously with AMD3100 and RA did not produce any RA enhanced neurite outgrowth that was previously observed. This result suggests that blocking CXCR4 signalling can inhibit the neurite outgrowth promoting effects of atRA.

Mean length of neurites from RA, CXCL12, and RA+CXCl12 treated explants were also significantly different ($p < 0.05$) from controls (Figure 10). AMD3100 had no significant effect ($p > 0.05$) on neurite length. The combination treatment of AMD3100

and RA did not have a significant effect on neurite length, supporting our previous result that blocking CXCR4 signalling can inhibit the growth promoting effects of atRA *in vitro*. Samples of spinal cold explants are shown in Figure 11.

These results indicate that normal chemokine signalling through CXCL12/CXCR4 is required for neurite outgrowth and extension *in vitro*. Blocking CXCL12/CXCR4 signalling can also inhibit the regeneration promoting effects of RA, which suggests that CXCR4 may act downstream of RARβ signalling during regeneration in the adult new spinal cord.

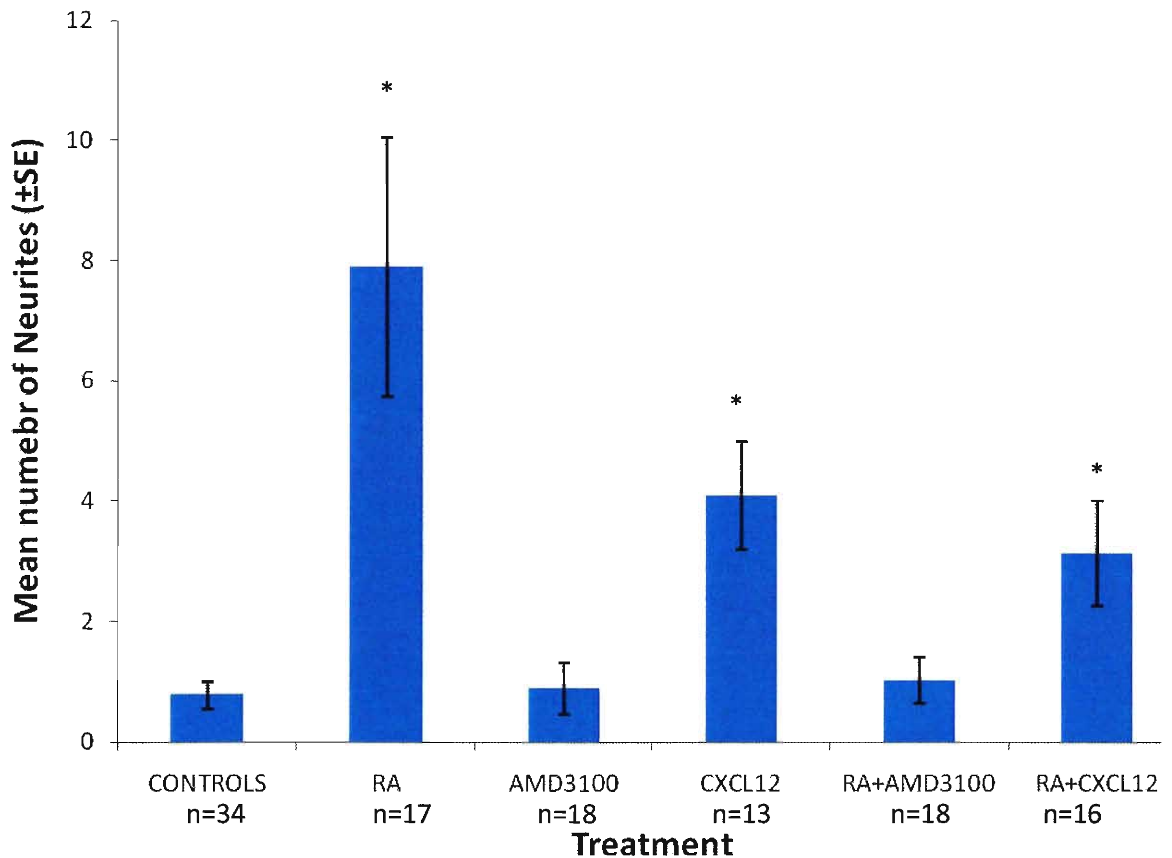


Figure 9. atRA and CXCR4 promotes neurite outgrowth from adult newt spinal cord explants (mean neurite number ±SE). Spinal cord explants were cultured in DM supplemented with specified treatments. There was no significant difference between control treatments (EtOH, PBS, EtOH+PBS), thus allowing data to be pooled into one control group. * Indicates a significant difference ($p < 0.05$) from controls. n indicates the number of whole explants that were analysed for neurite outgrowth.

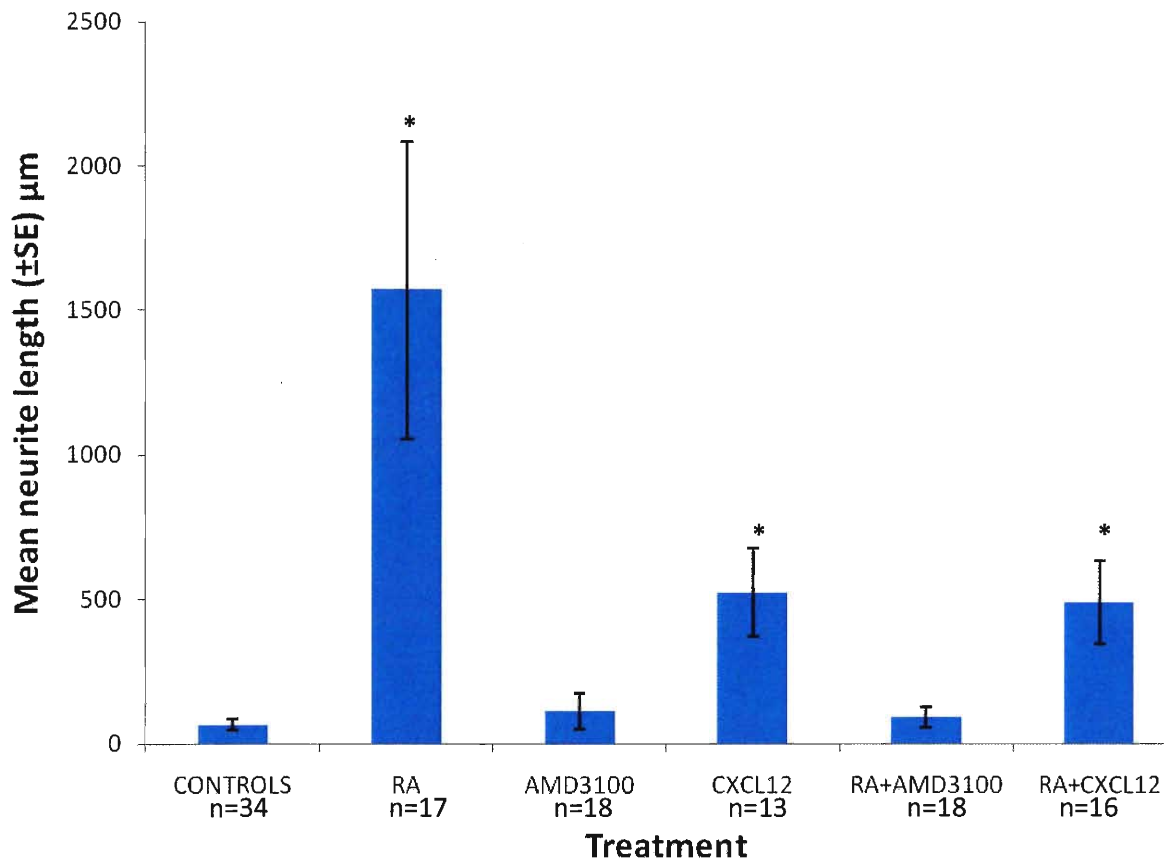


Figure 10. atRA and CXCR4 promotes neurite length from adult newt spinal cord explants (mean neurite length ±SE). Spinal cord explants were cultured in DM supplimented with specified treatemts. There was no significant difference between control treatments (EtOH, PBS, EtOH+PBS), thus allowing data to be pooled into one control group. * Indicates a significant difference ($p < 0.05$) from controls. n indicates the number of whole explants that were analysed for neurite length.

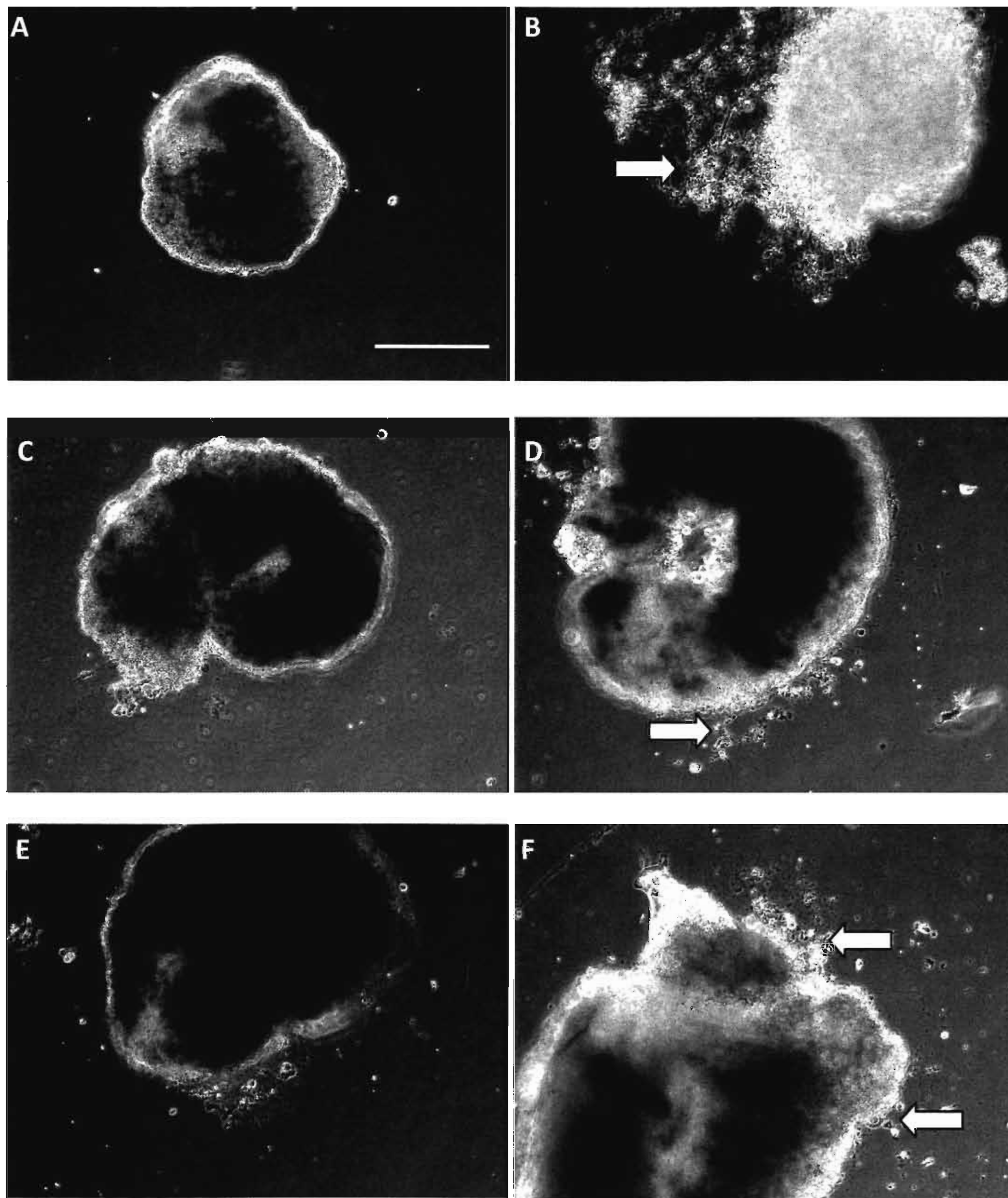


Figure 11. *In vitro* spinal cord explants display varying levels of neurite outgrowth depending on growth conditions. Culture conditions are as follows: A) EtOH control, B) RA, C) AMD3100, D) CXCL12, E) RA+AMD3100, F) RA+CXCL12. Scale bar = 50 μm. Arrows indicate areas of neurite/axonal outgrowth.

Chapter 5: Discussion

Previous studies have shown certain neural developmental pathways require functional CXCR4 signalling (Lieberam et al., 2005). Other significant pathways where CXCR4 is involved include migration of neural progenitor cells to sites of neural injury and inflammation in mammals (Imatola et al., 2004; Belmandi et al., 2006). These previous findings were observed in mammals, which are generally considered non-regenerating organisms. The roles of CXCR4 in the regenerating newt tail and spinal cord have not previously been investigated. Our observations show that CXCR4 is expressed in the regenerating newt tail and expression increases during the early phases of tail regeneration. CXCL12, the ligand of CXCR4, was also demonstrated to enhance neurite outgrowth from cultured adult newt spinal cord explants, and inhibition of CXCR4 by AM3100 abolished RA enhanced outgrowth in these cultures. Histological data also support a functional role for CXCR4 in the regenerating newt tail.

5.01 Newt CXCR4 Sequence Shows Regions of Homology with Other Species

My first objective was to clone a newt homologue of the CXCR4 receptor. Having done so, multiple alignment analysis of the CXCR4 receptor revealed specific regions of homology, and variability (Figure 3). The extracellular domain, particularly the N-terminus which is associated with ligand binding, displayed high variability across species (Zhou et al., 2001). In ligand binding studies of human CXCL12/CXCR4, three amino acids have been implicated to be necessary for binding CXCL12. N-terminal amino acids included Asp (D20A) and Tyr (Y21A). Glu (E268) in the third extracellular loop of CXCR4, was also involved in binding based on mutational studies. The specific amino

acids of the newt that interact with CXCL12 are not yet fully known. Opatz et al. (2008) used recombinant CXCL12, to stimulate outgrowth from rat dorsal root ganglia. Using the same CXCL12 ligand, we were also able to enhance outgrowth in new spinal cord explants. From this, we can hypothesize that the active site that is involved in binding is likely to be conserved across species and that the variations seen in the extracellular domain may not dramatically affect ligand binding.

The C-terminus and intracellular domains of CXCR4 are important for G-protein signalling and also regulating the internalization of the receptor. The C-terminus is subject to phosphorylation, which allows binding of regulatory proteins to downregulate CXCR4 signalling and internalization of CXCR4 with the appropriate signal (Berson and Doms, 1998), whereas other studies have found that the C-terminus positively regulated chemotaxis. For example, Minina et al. (2007) investigated further and found that internalization, and regulation of CXCL12 signalling levels mediated the migration of primordial germ cells. Since our sequence for *N. viridescens* shows higher homology at the C-terminal and intracellular domains to regions of CXCR4 in other animals (Figure 3), it is possible that the regulation of CXCR4 internalization during migration is accomplished in a similar fashion across species.

Crystallographic analysis of CXCL12 by Zhuo et al (2001) revealed that CXCL12 possesses a positively charged surface and binds to the ligand binding domain of CXCR4, which has a negatively charged surface. Various CXCR4 antagonists such as AMD3100 are also highly positively charged molecules. Thus it is possible that a similar mechanism

is being utilized by AMD3100 to inhibit newt CXCR4 based on sequence analysis and conserved domains.

5.02 CXCR4 Expression is Maintained in the Adult Newt Tail and Increases During Tail and Spinal Cord Regeneration

Our results show that CXCR4 is constitutively expressed in the adult central nervous system and all tested tissues of *N. viridescens.* The widespread expression of CXCR4 is not surprising due to the variety of roles that it plays in the body, such as in the immune system, which involves a global response in many tissues of living animals. During the development of the central nervous system, CXCL12/CXCR4 signalling is necessary in a wide variety of cells and cellular events such as cell migration, axonal guidance, branching, elongation, proliferation and cell survival (Arkawana et al., 2003; Chalasani et al., 2007; Imitola et al., 2004; Lieberam et al., 2005; Luo et al., 2006). The expression of CXCR4 in the adult mammalian nervous system has been reported in neurons, astrocytes, microglia, oligodendrocytes and endothelial cells (Bajetto et al., 2001; Lavi et al., 1997). This supports our findings that the highest levels of CXCR4 expression under normal physiological conditions in the newt are found in the brain. Imatola et al., (2000) reported that extensive CXCR4 expression was found in cerebellar glial, granule and Purkinje cells in the rat cerebellum. Cultured cells treated with CXCL12 demonstrated Ca^{2+} transients, increases in overall intracellular Ca^{2+} and spontaneous synaptic activity. They also reported that evoked excitatory action potentials could be reversibly reduced by applying CXCL12. This suggests that CXCL12/CXCR4 signalling has

an important neuromodulatory role in the adult CNS. CXCR4 has also been reported to promote neuronal survival in neuronal injury and inflammation (Khan et al. 2005). Nerveen et al. (2010) observed that atRA reduced the expression of various chemokines including CXCL chemokines in astrocytes, leading to a general suppression of the inflammatory response. Unfortunately CXCL12 was not specifically tested. The study corroborated with a previous finding that the immune response observed in mammals after injury is detrimental to regeneration and that a locally reduced immune response can help promote regeneration (Mescher and Neff, 2006).

Subsequent to tail and also caudal spinal cord amputation in the newt, we observed a steady increase in the expression of CXCR4 mRNA above basal levels detectable in the unamputated tail. At day 12 of regeneration we observed the highest level of expression for all stages analyzed. It is possible that this expression increased further at later stages, but they were not examined. There are many possible reasons for this increase in expression. First, there may likely be an immune response at the amputation site where a variety of immune cells would be required to prevent infection and help promote cell survival (Bluel et al 1996). It is also possible that CXCR4 could be involved in one or more processes during the early phase of spinal cord and tail regeneration. Some of the major events for functional regeneration to occur include dedifferentiation, cell proliferation, and redifferentiation, which occur in the ependymal response. CXCR4 has been shown to be involved in developmental pathways and evidence suggests that many of these pathways are recapitulated to a certain extent in

regeneration. For instance, Bouzaffour et al. (2009) reported that the Wnt and CXCL12 pathways interact during zebrafish fin regeneration to negatively regulate Fgf signalling in order to promote cell proliferation in a similar fashion as seen during development.

The increase in CXCR4 expression we detected also correlates with a period of increased cell proliferation, differentiation and neurogenesis in the regeneration tail of the adult newt (Benraiss et al., 1999). As early as day 6 post-amputation, neurons and glial cells have differentiated at the rostral end of the ependymal tube. This increasing population of neurons needs to be properly guided to their appropriate targets in the newly regenerated tissues. Assuming that CXCR4 functions in a similar way during regeneration as it does in development, regenerating neurites could express CXCR4 to ensure proper axonal guidance and innervation (Lieberam et al., 2005).

5.03 CXCL12/CXCR4 Signalling can Promote Neurite Outgrowth From Newt Spinal Cord Explants *in vitro*

These *in vitro* data demonstrated that treatment of newt spinal cord explants with CXCL12 can promote neurite outgrowth. Since our data also provide evidence that cells within the spinal cord of the adult newt express the receptor, CXCR4, it is possible that the increase in CXCR4 expression seen by day 12 post-amputation is required during the process of axonal guidance in the early phases of spinal cord and tail regeneration in the adult newt.

Explants were cultured on poly-L lysine substrate which provides a neutral growth environment for axons and is not considered to be inhibitory in the same fashion that a myelin substrate is. Opatz et al. (2008) reported that CXCL12 was also able to promote neurite growth from dorsal root ganglion cells in an inhibitory environment, which is also in accordance with the results of Chalasani et al. (2003). The latter demonstrated that CXCL12 removes the inhibitory effects of repulsive guidance cues by increasing the levels of cAMP (Chalasani et al., 2003). It would be an interesting future study to investigate if cAMP levels are elevated in adult newt spinal cord explants that are treated with CXCL12. It has also been reported that CXCR4 receptors are not evenly distributed throughout the cell and are highly concentrated at branching points and growth cones of axons providing further evidence to support sensory and motor axonal guidance roles of CXCR4 in regeneration (Opatz et al., 2008)

5.04 Inhibition of CXCL12/CXCR4 Signalling can Inhibit RA Induced Neurite Outgrowth in Adult Newt Spinal Cord Explants

RA has been previously shown to enhance and attract neurite outgrowth from newt spinal cord explants *in vitro* (Dmetrichuk et al., 2005). RA can also induce axonal growth cone turning in invertebrate neurons (Dmetrichuk et al., 2006). We have reproduced this RA enhanced outgrowth *in vitro*, moreover we were able to inhibit the outgrowth promoting effect of RA by blocking CXCR4 signalling. These results suggest a functional link between retinoid signalling and chemokine signalling in regeneration.

Yip et al. (2006) found that increased RARβ2 signalling in rat spinal cords could promote functional recovery after spinal cord injury. Similarly, Wong et al. (2006) reported that RARβ2 promoted the functional regeneration of sensory axons in the spinal cord. Using microarrays to perform differential gene profiling in rat spinal cords constitutively expressing RARβ2, it was observed that CXCR4 is downregulated (Yip, personal communication). In addition, Carmel et al. (2001) reported that CXCR4 is upregulated in injured rat spinal cords that are not constitutively expressing RARβ2. Due to these inverse relationships between RARβ expression and CXCR4 expression, one could hypothesize that CXCR4 is likely to contribute either directly or indirectly to prevent regeneration in the mammalian spinal cord. However this is not the case in urodeles.

Urodeles that can naturally regenerate their CNS, including the spinal cord, demonstrate different signalling relationships. At day 8 of regeneration, RARβ2 is upregulated in the newt spinal cord in relation to basal levels at day zero of regeneration (Carlone et al., 2006), however data presented here shows that CXCR4 expression is also upregulated at this time. The timing of peak expression levels of RARβ and CXCR4 in the regenerating spinal cord are not yet known, but this early increase in expression of both genes suggests that both may be required for functional regeneration in the newt spinal cord. Unlike RARβ2, which does not normally increase its expression levels in response to neural injury in mammals, CXCR4 expression increases in response to spinal cord injury in both mammals and urodeles. In

mammalian spinal cords, where RARβ2 can be constitutively expressed from a viral vector, CXCR4 appears to be down regulated. Normally, in the mammal, an increase in RARβ2 expression negatively affects CXCR4 expression. Thus we can assume that under normal circumstances, a decrease in RARβ2 expression can allow CXCR4 expression to increase in the mammal. However, in the mammal after injury, CXCR4 expression increases but it is not due to RARβ signalling. In the newt, RARβ2 is constitutively expressed (Carter, Personal Communication) as is CXCR4. As a consequence of injury, both increase in expression during the early stages of tail and spinal cord regeneration but not at the exact same time. The *in vitro* data presented here, along with other data from our laboratory (Carter, Personal Communication) provide evidence for a function link between RARβ2 and CXCR4 signalling. It is possible that CXCR4 expression in the newt is regulated by regeneration associated genes such as RARβ2 that may be acting up stream during regeneration.

Dufourcq et al. (2006) reported that CXCL12a and CXCR4a play critical roles in fin regeneration in zebrafish. CXCL12/CXCR4 expression was necessary for epidermal cell proliferation and the formation of the blastema which is the source of many regeneration signalling factors. At two days post-fin amputation, CXCL12 was expressed in the blastema and CXCR4 in the fin stump. If CXCRL12 was ectopically expressed by electroporation 16 hours before amputation, fin regeneration was completely abolished. It was suggested that the lack of regeneration was due to the inhibition of epidermal cell proliferation. Thus the location and timing of the CXCL12 signal is crucial

for regeneration to proceed normally. With regards to mammalian spinal cord ectopically expressing RARβ2, certain pathways that are not normally induced could have down regulated CXCR4 expression or perhaps delayed its expression during the regeneration process.

The roles of chemokine signalling, such as CXCL12/CXCR4, may vary between regenerating and non-regenerating vertebrates. In non-regenerating vertebrates, chemokine signalling is involved in the neuroimmune and inflammatory responses, which on one hand are considered to be inhibitory to spinal cord regeneration. The immune responses between mammals and urodeles also differ greatly. After spinal cord injury in urodeles, no astroglial scar is formed, and secondary injuries such as excitotoxicity seem to be avoided. Urodeles also have a low adaptive immunity which correlates with a higher regenerative ability. Furthermore, a depression in the immune response had been observed in regenerating urodele limbs (Mescher and Neff, 2006)

5.05 Altered Retinoid and Chemokine Signalling Affects Regeneration *in vivo.*

In regenerating tails implanted with RA soaked beads at 6 days post-amputation, we observed a down regulation in the expression of CXCR4 in the blastema at 9 days post-amputation. This suggests a regulatory link between RA signalling and CXCR4 signalling. This supports other work in rats where spinal cords ectopically expressing higher than normal levels of RARβ2 downregulate CXCR4 (Yip, Personal Communication). In this experiment, the level of retinoid signalling is well above normal physiological levels that is likely to have a variety of unknown effects. This does not

suggest that CXCR4 is detrimental to regeneration, but that RARβ2 signalling may have a modulatory role on CXCR4 signalling to regulate cell migration, proliferation, or axonal guidance during specific stages of regeneration.

In the mammalian CNS, the presence of adult neural stem cells or neural progenitor cells has been confirmed. These progenitor cells have been also found to express CXCR4 and have been observed to migrate under pathological conditions (Schwartz and Major, 2006). It is possible that neural progenitor cells in urodeles also express CXCR4 and migrate in a similar manner. Transplantation studies of injured mouse spinal cords with neural stem cells show that the stem cells migrated to sites of neural injury in a CXCL12 dependent manner. Cells at the injury site expressed CXCL12 and act as a homing beacon for the migrating neural stem cells. Stem cell migration correlated with peak CXCL12 expression at 7 days post injury (Imitola et al., 2004, Takeichi et al., 2007). The function of this stem cell homing process is not yet fully understood. This homing response could contribute to the inflammatory response that promotes neuroprotection and or regeneration, since the cells have been observed to differentiate into neurons and glial cells (Tanaka, 2003).

We have observed that CXCR4 is expressed in the adult newt spinal cord, but the location of CXCR4 expression in the surrounding tail tissues is unknown. This source of the CXCL12 signal in the regenerating newt tail is also not yet known, although the blastema would be a logical place since this tissue has been shown to provide chemoattractant molecules for neurites, such as atRA (Dmetrichuk et al., 2005). The

zebrafish fin provides a useful model for the roles of chemokine signalling during epimorphic regeneration. Various isoforms of CXCL12 and CXCR4 have been discovered to be involved in the regeneration process in the fish fin. CXCL12a was found to be localized in the blastema and CXCR4a in the stump epidermis. CXCR4b and CXCR7 were localized to the wound epidermis. Fgf and CXCL12 pathways have been previously shown to interact in development and also interact during regeneration which helps to carefully fine tune the regeneration process (Bouzaffour et al., 2009). Small changes in the expression of either pathway can severely affect or inhibit regeneration.

In the present study, regenerating newt tails implanted with AMD3100 soaked beads have altered the expression of CXCR4 mRNA. The regenerates with beads implanted at day 3 post amputation show a small increase in CXCR4 expression, while regenerates implanted at day 6 post amputation demonstrate downregulation in CXCR4 expression (Figure 6). It is possible that earlier in regeneration, the blastema cells are compensating for blocked CXCR4 signalling by upregulating mRNA expression. At later stages of regeneration, the opposite trend is observed. This suggests that at different time points of regeneration, compensatory mechanisms may be functioning to promote regeneration. Later time periods, day 6 post amputation, appear to be more sensitive to the inhibition of CXCR4 signalling than do earlier time periods (day 3), producing the histological changes that were observed. Without replication of the data, it is difficult to draw solid conclusions. Also, that rate, and amount of agent being released from thee beads is unknown. Thus, further investigation is required.

More currently in our lab, variations of these experiments have been repeated. It has been observed that inhibiting CXCR4 signalling with AMD3100 produced the greatest effect if the beads were implanted zero days post amputation. Using this technique, tail regeneration was significantly impaired (Clarke, Personal Communication). This new data supports my initial evidence that altered CXCR4 signalling is detrimental to tail regeneration.

5.06 Inhibition of CXCR4 Signalling Inhibits Extension of the Ependymal Tube During the Early Phases of Regeneration

Inhibition of CXCR4 signalling by implanting beads soaked with AMD3100 appears to delay regeneration or inhibit it. Normally regenerating tails at day 14 of regeneration show extension of the ependymal tube into the blastema. Here we did not see an ependymal extension (Figure 7). The only sign of an ependymal response was the presence of the ependymal bulb at the distal end to the amputated spinal cord which appeared to have retreated slightly into the tail stump. This impairment of the ependymal response was also seen by Tassava and Huang (2005) where ischemia was artificially produced in amputated newt tails. Regeneration was severely delayed or inhibited completely under ischemic conditions, which also resulted in spinal cord and ependymal breakdown. In ischemic tails, a pseudoblastema was observed. A pseudoblastema appears similar to a blastema in that it has a wound epithelium and dedifferentiated cells. The wound epithelium is thicker in the pseudoblastema, however, the size of the blastema is relatively smaller and contains fewer cells. There is also little

or no outgrowth of the ependymal tube (Tassava and Huang, 2005). These characteristics are similar to our observations in regenerating tails treated with AMD3100 beads. Once again this set of results are only preliminary but it appears that blocking the CXCR4 receptor during regeneration is detrimental to regeneration.

5.07 General Conclusions

In this study we have expanded our knowledge of the coding sequence of *Notophthalmus viridescens* CXCR4 and have observed that CXCR4 is expressed in the adult newt tail and spinal cord, as well as in other tissues such as the heart, gut, and liver. In the regenerating newt tail, the expression of CXCR4 increases during the early stages of regeneration and returns to basal levels by day 21 of regeneration. Functional studies provide evidence that CXCL12/CXCR4 signalling is able to promote neurite outgrowth from spinal cord explants *in vitro*. Also, *in vivo* studies using AG1X2 beads suggest that inhibiting CXCR4 signalling delays or inhibits proper tail regeneration of the newt tail and spinal cord. However, these *in vivo* results are only preliminary. *In vitro* evidence suggests a functional link between retinoic acid signalling and CXCL12/CXCR4 signalling where the inhibition of CXCR4 signalling abolished retinoic acid enhanced neurite outgrowth. Taken together these data suggest that CXCR4 signalling may act as a downstream effector pathway of RARβ signalling and that CXCR4 is an important factor in spinal cord and tail regeneration in *N. viridescens.*

Chapter 6: References

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Chapter 7: Appendix

A

5’AGGTGAAGAAGGTGAGGATGAGAATCACCGTCGTCTTCAATGCTTTGCGCTTCTGGTGGCCT
TTGGAGTGGGACAGCCTAGAAATTATGATGCAGTAGCAAATCAAGATGACCAGGCCAGGCAGA
ACAAACCCTACCGTGAGGTGCACAAACCTAAATCCAACAGTCCACTCTTTCCGCGTCTCGTAGG
GATAGATGTGGTTGCATTCGTAGGTGCCTTCTTCCATACTAACACTGGCAAATATGAAGTCGGG
CACGGTCAAGAGCACAGCCGGCAACCACACCCCAGCATACACCACCTTCTCAGCCAGCAGCTTC
CTCGTACCTTGGCTGTTGGTGGCGTGGACTATCGCCAAGTAGCGGTCTAAACTTATGAAGGCCA
AGATCAG 3’

B

LILAFISLDRYLAIVHATNSQGTRKLLAEKVVYAGVWLPAVLLTVPDFIFASVSMEEGTYECNHIYPYET
RKEWTVGFRFVHLTVGFVLPGLVILICYCIIISRLSHSKGHQKRKALKTTVILILTFFT

Figure A1. Original partial sequence of CXCR4 from *N. viridescens.*

(A) 5’ to 3’ partial cDNA sequence.

(B) Partial amino acid sequence.

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N. v  240  TCAAGGACTATGACGGACAAGTACCGGCTGCACCTGTCTGTGGCCGATCTTCTGTTTGTC  299
           |||||||||||||| ||||||||||||||||||||||| ||||||||||| |||||||||
T. m  2    TCAAGGACTATGACCGACAAGTACCGGCTGCACCTGTCGGTGGCCGATCTGCTGTTTGTC  61

N. v  300  TTCACCTTGCCCTTCTGGTCCGTAGATGCTGTGATAGGCTGGTGTCTTAAAGAGTTCCTC  359
           ||||||||||||||||||||||||||||| |||||||| |||| ||| || |||||||||
T. m  62   TTCACCTTGCCCTTCTGGTCCGTAGATGCCGTGATAGGGTGGTATCTGAAGGAGTTCCTC  121

N. v  360  TGCAAAGCCGTTCACGTCATCTACACAGTCAACCTCTACAGCAGCGTCCTGATCTTAGCC  419
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
T. m  122  TGCAAAGCCGTTCACGTCATCTACACAGTCAACCTCTACAGCAGCGTCCTGATCTTAGCC  181

N. v  420  TTCATAAGTTTAGACCGCTACTTGGCGATAGTCCACGCCACCAACAGCCAAGGTACGAGG  479
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
T. m  182  TTCATAAGTTTAGACCGCTACTTGGCGATAGTCCACGCCACCAACAGCCAAGGTACGAGG  241

N. v  480  AAGCTGCTGGCTGAGAAGGTGGTGTATGCTGGGGTGTGGTTGCCGGCTGTGCTCTTGACC  539
           |||||||||||||||||||||||||| ||||||||||||||||||||||||||| |||||
T. m  242  AAGCTGCTGGCTGAGAAGGTGGTGTAYGCTGGGGTGTGGTTGCCGGCTGTGCTCCTGACC  301

N. v  540  GTGCCCGACTTCATATTTGCCAGTGTTAGTATGGAAGAAGGCACCTACGAATGCAACCAC  599
           ||||| |||||||||||||||||||||||||||||||||||||||||||| |||||||||
T. m  302  GTGCCTGACTTCATATTTGCCAGTGTTAGTATGGAAGAAGGCACCTACGAGTGCAACCAC  361

N. v  600  ATCTATCCCTACGAGACGCGGAAAGAGTGGACTGTTGGATTTAGGTTTGTGCACCTCACG  659
           ||||||||||  ||| ||||| || |||||||||||||||||||||||||||||||||| 
T. m  362  ATCTATCCCTTTGAGTCGCGGCAACAGTGGACTGTTGGATTTAGGTTTGTGCACCTCACC  421

N. v  660  GTAGGGTTTGTTCTGCCTGGCCTGGTCATCTTGATTTGCTACTGCATCATAATTTCTAGG  719
           ||||||||| ||||||| |||||||||||||||||||||||||||||||| ||||| | |
T. m  422  GTAGGGTTTATTCTGCCCGGCCTGGTCATCTTGATTTGCTACTGCATCATCATTTCCAAG  481

N. v  720  CTGTCCCACTCCAAAGGCCACCAGAAGCGCAAAGCATTGAAGACGACGGTGATTCTCATC  779
           |||||||||||||||||||| ||||||||||||||  |||||||||| ||||||||||||
T. m  482  CTGTCCCACTCCAAAGGCCAACAGAAGCGCAAAGCGCTGAAGACGACTGTGATTCTCATC  541

N. v  780  CTCACCTTCTTCACCTGCTGGTTGCCCTACTACATCGCCCTCAGCATAGACACCTTCATA  839
           |||||||||||||||||||||||||||||||||||| |||||||||| ||||||||||||
T. m  542  CTCACCTTCTTCACCTGCTGGTTGCCCTACTACATCACCCTCAGCATTGACACCTTCATA  601

N. v  840  CAAATGGGCGTGATCAAGAACATTACATGTGACTTT-GAGGGCACTCTGGGTACGTGGAT  898
           ||| |||||||||||||||||||||||||||| ||| |||| ||| |||  |||||||||
T. m  602  CAACTGGGCGTGATCAAGAACATTACATGTGA-TTTCGAGGTCACCCTGCATACGTGGAT  660

N. v  899  CTCCATCACTGAAGCCTTGGCCTTCTTCCACTGCTGCCTGAACCCCATCCTCTAC  953
           |||  ||||||||||||||||||||||||||||||||||||||||||||||||||
T. m  661  CTCYGTCACTGAAGCCTTGGCCTTCTTCCACTGCTGCCTGAACCCCATCCTCTAC  715
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Figure A2. BLAST analysis of full length CXCR4 cDNA sequence.
N. v *(N. viridescens),* T. m *(Triturus marmoratus). Triturus marmoratus* chemokine receptor 4 (CXCR4) gene, partial cds (gb|EF107448.1|). Identities = 677/715 (94%),Gaps = 2/715 (0%). Strand=Plus/Plus