INVOLVEMENT OF THE ENDOCANNABINOID SYSTEM IN THE EPENDYMOGLIAL RESPONSE TO SPINAL CORD REGENERATION IN THE MEXICAN AXOLOTL, *Ambystoma mexicanum*

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Abstract

Research into the molecular mechanisms of the psychoactive effects of cannabis has led to the discovery of the endocannabinoid system (ECS), a neuromodulatory system conserved throughout the animal kingdom. Little is known about its function in mammals, but there is evidence suggesting its contributions in the cellular processes that are important in CNS development and are conserved during CNS regeneration. However, these studies focussed primarily on mammals, which display limited abilities to regenerate after traumatic CNS injury. Furthermore, nothing is known regarding the role of endocannabinoids in CNS regeneration-competent species like the Mexican axolotl, one of the few vertebrates that can regenerate their spinal cord. The current study investigates the potential role of the ECS in influencing the pro-regenerative response observed in the axolotl spinal cord. I provide evidence that the main ECS receptor in the CNS (CB1) is upregulated in the regenerating caudal spinal cord and tail tissues of larval axolotls at 4 hours post amputation, lasting until 14 days post amputation. By performing immunofluorescence studies on these tissues, I demonstrate the expression of this receptor mainly in the ependymal region. In addition, bath application of the CB1 inverse agonist, AM251, significantly inhibited caudal growth of the spinal cord and tail by 7 days post amputation. The current study also identified an upregulation in a second ECS receptor, CB2, at 7- and 14-days post amputation. Immunofluorescence analysis revealed the localization of this receptor to the subependymal regions within the spinal cord. Furthermore, inhibition with the CB2 inverse agonist, AM630, similarly demonstrated an inhibition in spinal cord and tail regeneration by 7 days post amputation. An assessment of CB1 and CB2 expression was performed by identifying their localization in

bromodeoxyuridine-positive (proliferating) and doublecortin-positive (differentiating neuronal) cells in 7-day regenerate tissue. These studies are the first to examine the role of the ECS during spinal cord regeneration in a regeneration-competent vertebrate and may aid in developing novel therapies for human nervous system injuries or pathologies.

Keywords: Regeneration, Axolotl, Ependymoglial, CB1, CB2

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List of Abbreviations

2-AG, 2-Arachidonoylglycerol

ACEA, Arachidonyl-2'-chloroethylamide

AEA, Anandamide

AM251, 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide

AM630, [6-iodo-2-methyl-1-(2-morpholin-4-ylethyl)indol-3-yl]-(4-methoxyphenyl)methanone

BMP, Bone morphogenic protein

BrdU, Bromodeoxyuridine

cAMP, Cyclic AMP, or 3',5'-cyclic adenosine monophosphate

CB1, Cannabinoid receptor type-1

CB2, Cannabinoid receptor type-2

CC, Central canal

CCI, Chronic constriction injury

CNS, Central nervous system

DAGL, Diacylglycerol lipase

DAMP, Damage-associated molecular patterns

DCX, Doublecortin

DPA, Days post amputation

D/T80, DMSO:Tween-80 vehicle control

ECS, Endocannabinoid system

EG, Ependymoglial cell

ERK, Extracellular signal-regulated kinase

FAAH, Fatty acid amide hydrolase

GABA, γ-aminobutyric acid

GFAP, Glial fibrillary acidic protein

GPCR, G protein-coupled receptor

HPA, Hours post amputation

JWH-133, (6aR,10aR)-6,6,9-trimethyl-3-(2-methylpentan-2-yl)-6a,7,10,10a-tetrahydrobenzo[c]chromene

MAPK, Mitogen-activated protein kinase

NAPE-PLD, N-arachidonylphosphatidylethanolamine-phospholipid D

NG2, Oligodendrocyte progenitor cell

NOGO, Neurite outgrowth inhibitor

NPC, Neural progenitor cell

NSC, Neural stem cell

MAGL, Monoacylglycerol lipase

pERK, Phosphorylated-extracellular signal-regulated kinase

SE, Subependymal cells

SHH, Sonic hedgehog

SNL, Spinal nerve ligation

SVZ, Subventricular zone

SGZ, Subgranular zone

TRPV1, Transient receptor potential cation channel subfamily V member 1

UINJ, Uninjured tissue

Chapter 1. Introduction and Literature Review

1.1 General introduction

Regeneration has been an interest in the scientific community for centuries. It was during the 18th century when we began to understand and develop the theories behind the events that take place during this phenomenon (Tsonis & Fox, 2009). The notable discoveries during this time included studies on regenerating insect appendages by Réaumer in 1712, and in the regeneration of hydra and worms in 1744 by Tremblay and Bonnet. However, it was in 1768 when Spallanzani introduced some of the first studies detailing the regeneration of tetrapods, which sparked the investigations into organisms with anatomies somewhat resembling humans (Dinsmore, 1996). This included urodele amphibians (axolotls, newts, and salamanders) as he details his observations following limb and tail amputation to fully functional regenerates. When it came to tail amputations in salamanders, Spallanzani documented the rapid blood flow and skin retraction in the injured area in order to develop the wound epithelium (Tsonis & Fox, 2009). He then described what we currently title the blastema from the outward growth of dividing cells that appeared in this area following injury (Tsonis & Fox, 2009). From these initial observations, we would soon learn that the molecular and cellular events in response to injury differ between vertebrates and may enable or inhibit their ability to achieve successful and functional regeneration.

Since these discoveries, scientists have continued to pay more attention to the differing regenerative capacities across the animal kingdom (Godwin & Rosenthal, 2014). Notably, when the central nervous system (CNS) is subjected to damage, the ability of neurons in the brain and/or spinal cord to find their corresponding synaptic partner(s) and

allow for functional repair is dependent on the injured organism's regenerative capacity. Such processes are limited in mammals and damage to the CNS can often result in permanent paralysis, as outgrowth of functional axons from neurons is impeded by negative interactions with glial cells, and/or the formation of a glial scar (Yu & Bellamkonda, 2001). Urodele amphibians (e.g., the newt and axolotl) are excellent models to investigate regeneration for their extensive regenerative abilities in various tissues (Godwin & Rosenthal, 2014). In particular, the Mexican axolotl, *Ambystoma mexicanum*, possesses the capacity to regenerate its spinal cord and portions of its brain (Amamoto et al., 2016). The complete transection of the axolotl spinal cord induces rapid proliferation of a population of neural stem cells (NSCs) called ependymoglial cells. NSCs are a class of immature cells that are able to proliferate, self-renew, and produce a neurons and glia (Temple, 2001). These cells are reported to be the main contributors to spinal cord regeneration and will extend toward the wound to form the ependymal tube and bulb at the site of injury where these cells will contribute as new neurons and glial cells (Chernoff et al., 2018). The factors involved in the establishment of a permissive environment for CNS regeneration in axolotls are yet to be fully understood, but there have been various cell signaling systems suggested to contribute to this process (Mchedlishvili et al., 2012).

A promising signaling system that has yet to be examined is the endocannabinoid system (ECS). There have been studies to suggest a neuroprotective role of the ECS after CNS trauma in mammals (Arevalo-Martin et al., 2012), and identifying its presence in the astrocytic domains of mammalian spinal cords (Torija et al., 2015). However, the ECS is relatively unexplored in the context of regeneration in the CNS of regeneration-competent vertebrates. It is well documented in mammalian models that the enzymes involved in the

production and degradation of ECS ligands as well as cannabinoid receptor expression are present in, and help regulate, the NSCs within the ependymal region of the spinal cord and other neurogenic niches during development (Díaz-Alonso et al., 2012; Garcia-Ovejero et al., 2012; Paniagua-Torija et al., 2015). In addition, disruption of CB1 signaling in early zebrafish development has led to impaired cortical generation of the neurons from NSCs (otherwise known as neurogenesis), neural progenitor proliferation, and migration (Oudin et al., 2011; Watson et al., 2008). Such processes, which occur normally during CNS development, also play a vital role in regeneration.

The ECS involves two main receptors subtypes, the cannabinoid type-1 (CB1) and type-2 (CB2) receptors (Pertwee, 1997). The CB1 receptor is the main receptor subtype in the CNS, but CB2 appears to be more widely distributed and is not confined to the CNS (Elphick & Egertova, 2001; Cabral & Griffin-Thomas, 2009). Most studies have demonstrated that it is mainly expressed in immune cells and largely within the spleen and lymphatic system. However, CB2 has recently been identified in the neurons and glia of the CNS in rodents and has been of interest due to its inducible nature in neuroinflammatory events (Palazuelos et al., 2008; Bu et al., 2016; Jordan & Xi, 2019).

CB1 activation is induced by binding of endogenous cannabinoids (i.e., endocannabinoids). The most well-known endocannabinoids are anandamide and 2-arachidonylglycerol (2-AG), which are both synthesized in the CNS (Dunham et al., 2012). The ECS is active during neurodevelopment in processes including axonal guidance, synapse formation, and cell proliferation in vertebrates (Aguado, 2006; Arafah et al., 2013). However, little information has been gathered about its modulatory roles in response to CNS damage. Based on its putative roles in neural development, it is not unreasonable to

suggest that the ECS may be a novel signaling pathway to mediate the cellular events providing a permissive environment for regeneration in regeneration-competent vertebrates such as the axolotl. Therefore, the aim of my research was to examine the involvement of the ECS in regulating the fate decisions of ependymoglial cells to either self-renew as stem cells or differentiate as neurons or glia to ultimately contribute to the regenerated caudal spinal cord and tail of the axolotl.

1.2. The cellular response to spinal cord injury in mammals

Mammalian regeneration in the context of the CNS has long been researched for its potential biomedical applications. The CNS is comprised of the brain and spinal cord and is composed of a network of numerous cell types, which include neurons, astrocytes, microglia, and oligodendrocytes (Adams & Gallo, 2018). In comparison to other organisms, mammals appear to lack the capacity to regenerate and functionally heal following injury to the CNS. This feature has been attributed to particular intrinsic and extrinsic factors in the site of injury (Vajn et al., 2013). Macroscopically, an injury to the CNS will cause Wallerian degeneration caudal to the lesion and the formation of a "retraction bulb" rostral to the injury (Vajn et al., 2013). These are structures characterized as swellings and nongrowing portions of growth cones, the highly dynamic and actin rich structures extending from regenerating or developing neurites (Strochlic et al., 2007), resulting in microtubule network disorganization, which disrupts normal axonal outgrowth (Erturk et al., 2007). By disrupting the ascending and descending neuronal fibre tracts of the spinal cord, major motor and sensory dysfunction results, which can negatively impact the motility, continence, and overall quality of life of a person (Shigyo & Tohda, 2016). It

is for this reason that understanding the molecular events that underly the cellular responses to create an environment conducive to spinal cord regeneration is of interest today.

When an injury to the spinal cord occurs, as with other areas of the CNS, a structure known as the glial scar is formed. Scarring appears to be a cellular response exhibited by various tissues as a means to limit the extent of damage, and to potentially restore that tissue subsequent to CNS injury (Bradbury & Burnside, 2019). This response is regulated by intrinsic factors within the cells local to the wound and the interactions with external factors in the extracellular matrix (Vajn et al., 2013; Bradbury & Burnside, 2019). In the context of spinal cord injury in mammals, it is believed that a combination of factors in the extracellular matrix and cellular factors function in concert to prevent spontaneous regrowth of neuronal axons (Vajn et al., 2013).

The glial scar in mammals has been studied in a variety of contexts such as, contusive injuries, sharp penetrating trauma, and ischaemic lesions. A main contributor to the glial scar is reactive gliosis, which is the process where the glia local to this region of CNS injury or pathology undergo hypertrophy and hyperplasia (Eng et al., 1987). In the spinal cord, an injury initiates a cascade of events that impact the cells at the site of injury. This involves various classes of glia such as, astrocytes, NG2 glia, and microglia, as they develop a physical barrier in the injured area. This forms an area of the scar known as the lesion core (Figure 1), which is home to perivascular fibroblasts, pericytes, ependymal cells, phagocytic macrophages, and matrix proteins that inhibit axon regeneration (Adam & Gallo, 2018). During this process, astrocytes are directed to this gliotic response due to molecules that elicit inflammatory responses such as, danger-associated molecular patterns (DAMPs) and both proinflammatory cytokines and chemokines that are released by

microglia and astrocytes when the injury occurs. These astrocytes will then populate the borders of the injury and they strongly upregulate the expression of intermediate filament proteins such as, glial fibrillary acidic protein (GFAP), nestin, and vimentin (Sofroniew, 2014; Zamanian et al., 2012). This leads to elongation and extension of overlapping astrocytic processes, resulting in a barrier-like structure (Wang et al., 2012). The glial scar is heterogeneous; it has contributions from fibroblast-like cells, oligodendrocyte precursor cells, microglia, and adaptive immune cells, but astrocytes have been given the most attention for their reactive response to injury (Bradbury & Brunside, 2019). Extracellular factors within the glial scar have also been widely studied. These factors include oligodendrocyte and myelin-derived factors such as neurite outgrowth inhibitor (Nogo) A, myelin associated glycoproteins (MAGs), oligodendrocyte myelin glycoproteins (OMgps), as well as chondroitin sulfate proteoglycans (CSPGs). All of these factors have been studied for their inhibitory or abortive actions on axonal regeneration and plasticity in the scar environment (Vajn, 2013; Bradury & Brunside, 2019).

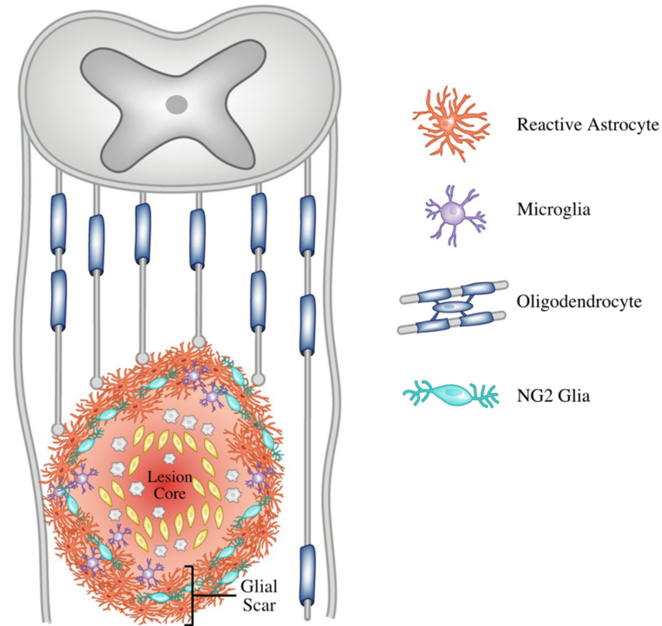


Figure 1. Illustrated schematic of the cellular components of the glial scar. This diagram is modified from Adams & Gallo (2018) and illustrates the glial scar after spinal cord injury. Injured axons (gray fibers) are unable to grow through the glial scar. The glial scar is composed of reactive astrocytes NG2 glia, and microglia formed around the site of injury known as the lesion core. The lesion core contains blood-borne macrophages (gray) and stromal cells (yellow).

The glial scar is also an interest in the scope of spinal cord injuries because of its controversial roles described in the literature. It was previously believed that the glial scar provided an exclusively inhibitory environment for damaged spinal cords (Fawcett & Asher, 1999). This is due to the findings previously mentioned involving the creation of an impenetrable physical barrier to axonal growth and the inhibitory signals to regenerating axons. However, beneficial roles of the glial scar have also come to light. Various rodent and other mammalian studies have demonstrated that preventing the formation of the glial scar does not always lead to increased spinal cord regeneration and recovery (Anderson et

al., 2016). Furthermore, preventing scar-forming astrocytes from performing their reactive function has led to findings showing axonal retraction and more severe phenotypes such as neuronal cell death and demyelination (Anderson et al., 2016; Filous et al., 2014). Mice knock-out studies of the intermediate filament proteins involved in the glial scar, GFAP and vimentin, produced a smaller glial scar following injury, but resulted in increased haemorrhaging, fibrosis, and accumulation of cellular debris (Pekny et al., 1999). This demonstrates the diverse roles of the glial scar during spinal cord injuries and creates an interest in investigating the events and potential differences in the response of astrocytes and the formation and composition of the glial scar in organisms that do exhibit functional recovery from spinal cord injuries, such as the Mexican axolotl.

1.3. Spinal cord regeneration in the axolotl

As mentioned, mammals possess various limitations to successful and functional spinal cord regeneration. Such limitations include the absence of solitary growth pathways for regenerating axons, inhibitory molecules that are native to the site of injury and within the glial scar, and a lack of, or decreasing population of adult NSCs that can create new neurons following injury (He & Jin, 2016; Silver et al., 2014; Tanaka & Ferretti, 2009). One of the organisms that has retained this ability to regenerate various components of the adult CNS is *Ambystoma mexicanum*, the Mexican axolotl.

To study spinal cord regeneration in the axolotl, tail amputations, partial spinal cord lesions (hemisections), complete spinal cord lesions (transections), and spinal crush injury paradigms have all been performed (Butler & Ward, 1965; Piatt, 1995). Of these methods, the most common forms of spinal cord injury performed in labs are thoracic spinal cord transections and tail amputations (Tazaki et al., 2017). Although the cellular and pro-

regenerative response to injury in these two locations and injury paradigms are comparable (O'Hara et al., 1992), the main differences include the unipolar growth of the spinal cord after tail amputations and the bipolar growth to connect both the caudal and rostral ends of the spinal cord observed in transections (Tazaki et al., 2017). Tail injuries have shown to be advantageous in some respects due to faster regeneration, greater efficacy in live imaging the regeneration process, and ease for electroporation protocols (Tazaki et al., 2017).

After tail amputation, a process known as epimorphic regeneration will occur. During this process, a blastema, a collection of undifferentiated cells that populate the area of injury, is formed to lead to the regeneration of the lost tissue (Freitas et al., 2019). The blastema contains cells of various lineages including muscle tissue, connective tissue, and nervous tissue. The blastema also contains various progenitor cell and NSC populations that will aid in the regeneration of the new caudal spinal cord (Freitas et al., 2019).

During the regenerative response, it was documented early on that the axolotl is able to fully regenerate their nerve fibres across a spinal cord lesion (Goss, 1969). However, Goss also suggested that new cells must be generated in the spinal cord during regeneration to provide for the rebuilding of the lost structures. The cells that are responsible for this are derived from the embryonic radial glial cells during development and are classified as the ependymoglial cells. These are thought to act as the NSCs that border the central canal in the spinal cord (Chernoff, 2003; Joevn & Simon, 2018). Amongst the glia, the ependymoglial cells are class of radial glia and are the most abundant and maintain morphological features similar to those found in NSCs in the developing spinal cord (Tazaki et al., 2017).

Ependymoglial cells are known to play a vital role in regeneration. After spinal cord injury, the amputated end of the cord will be sealed by ependymal radial glia cells and a blastema will form (Rodrigo-Albors et al., 2015). These stem cells will increase the rate of their cell cycle progression 4-fold, entering a highly proliferative state that is coupled to symmetric divisions that allow for rapid expansion of the stem cell pool. This rapid expansion is in contrast to the normal asymmetric neurogenic divisions seen in these stem cells, where they each produce one neuron and stem cell (Rodrigo-Albors et al., 2015). This shift to symmetric division leads to the rapid proliferation of these stem cells and development of an "ependymal tube", a caudal tube-like extension composed of ependymoglial cells. The cells within this tube have migratory properties that contribute to the outgrowth from the stump tissue. These stem cells will then differentiate into a variety of neurons and glial cells that are required to replace the lost tissue (Tazaki et al., 2017). In addition, axons that are rostral to the site of injury will elongate and descend caudally to aid in forming the new spinal cord (Zhang et al., 2003). Thus, successful regeneration of the spinal cord is dependent upon the migration and proliferative response of the ependymoglial cells.

The molecular events that mediate these behaviours of ependymoglial cells and the events required for spinal cord regeneration are not entirely understood. Various signaling pathways are shared between development and regeneration as many events that take place in regeneration are recapitulated from development. These pathways include, but are not limited to, retinoid signaling (Hunter, 1991), wnt signaling (Caubit, 1998), sonic hedgehog (SHH) signalling (Schnapp et al., 2005), Bone morphogenic protein (BMP) signaling (Beck, 2006), and Axolotl Marcks-like protein signaling (Suguira et al., 2016). These

pathways are similar in that they all mediate events important for normal spinal cord development. Given this information, it is believed that axolotl ependymoglial cells retain the developmental patterning systems, cell competence, and spatial organization during regeneration and throughout their lifespan.

One developmental signaling pathway that has only recently drawn interest with respect to a potential role in CNS regeneration is the endocannabinoid system (ECS). Cannabinoids have recently been demonstrated to be involved in the NSC niches within the spinal cord and have previously been identified in the astrocytic domains of the ependymal regions in the rat and human spinal cord (Paniagua-Torija et al., 2015). Furthermore, various glial cells within the spinal cord have exhibited the machinery required for endocannabinoid synthesis, degradation, and transport. It is currently believed that the ECS could potentially contribute to both neuroprotective responses and anti-inflammatory effects (Massi et al., 2008). Indeed, these results indicate that successful spinal cord regeneration is a highly choreographed response of the cellular events to coordinate growth, patterning, and differentiation. Given the recent data involving the endocannabinoid system and glial cells, it might suggest there is a role for endocannabinoids in the regenerative response in the axolotl spinal cord.

1.4. The endocannabinoid system (ECS)

The first documentation of the use of cannabis as medicine dates back to 2737 BCE in China and is now one of the world's most highly used psychoactive substances (Mechoulam, 1986; WHO, 2020). The active metabolite that is responsible for the psychoactive effects associated with cannabis was first isolated in 1964 and is now known as Δ^9-tetrahydrocannabinol (THC) (Gaoni & Mechoulam, 1964). Since then, the research

into the molecular mechanisms underlying the psychoactive effects of cannabis has led to the identification of the receptors that THC acts upon and eventually the endogenous cannabinoid system (endocannabinoid system or ECS) (De Petrocellis et al., 2009). The ECS is a lipid-based signaling system that performs neuromodulatory functions in the vertebrate central and peripheral nervous systems and within invertebrate nervous systems (Pacher et al., 2011).

The ECS involves two main receptors that belong to the G-protein coupled receptor (GPCR) family, the cannabinoid receptors type-1 (CB1) and type-2 (CB2). Studies involving genetic deletions of the classical cannabinoid receptors suggests there are other targets for the ligands of the ECS (Howlett, 2002). These have been categorized as non-CB1/CB2 receptors (Brown, 2007); however, much less research has been performed on these targets. Thus, the remainder of this chapter will focus on CB1 and CB2. These receptors have acquired their name for their affinity for THC (Howlett, 2002). The CB1 receptor is the main receptor subtype in the CNS where it has been identified in the spinal cord and regions throughout the brain, such as the cerebral cortex, hippocampus, basal ganglia, and the cerebellum (Elphick & Egertova, 2001; Herkenham et al., 1990). Furthermore, these receptors are predominantly expressed presynaptically on axons and axon terminals (Salio et al., 2002; Nyíri et al., 2005; Farquhar-Smith et al., 2000); however, there are also reports of postsynaptic neuronal expression and glial expression (Rodríguez et al., 2001). External to the CNS, there are also reports of CB1 within peripheral neurons, adipocytes, human adrenal glands, lung tissue, skeletal muscle, colonic tissue, and selective reproductive organs (Reggio, 2010). Alternatively, CB2 was initially documented in peripheral immune cells and organs. This includes the spleen, thymus, tonsils, mast cell

populations, and macrophage populations (Howlett, 2002). Despite the previous belief that CB2 was absent within the CNS, neuronal and microglia expression of CB2 is now widely accepted (Atwood & Mackie, 2010). For the basis of the current study, the roles of CB1 and CB2 in the context of the CNS will be the focus for the remainder of this chapter.

These receptors belong to the Class-A GPCR family and are amongst the most abundant GPCRs in the brain (Mackie, 2008; Reggio, 2010). Signaling through these receptors is primarily through the inhibitory G proteins, Gi and Go, but there are reports of Gs and Gq/11 signaling under certain conditions (Freund et al., 2003; Howlett et al., 2002). Activation of the receptors associated with inhibitory G-proteins will inhibit adenylyl cyclase, which reduces the production of cyclic adenosine monophosphate (cAMP) and results in modulating mitogen-activated protein kinases, Protein Kinase A, and Protein Kinase B (Dalton & Howlett, 2013; Dalton et al., 2009; Zou & Kumar, 2018). In addition, CB1 activation inhibits particular voltage-gated calcium channels and activates G-protein linked inwardly rectifying potassium channels (Mackie, 2008; Howlett, 2002). Together, the expression of these signaling pathways on presynaptic terminals suggests a role for CB1 stimulation in suppressing neuronal excitability and inhibition of neurotransmission (Freund et al., 2003). There are additional reports that similarly demonstrate CB1-mediated cAMP production through Gs proteins when coupled to dopamine receptor 2 stimulation in striatal neurons (Glass et al., 1997). Although GPCRs, CB1 and CB2 are primarily found localized in the cell membrane, there is increasing evidence for intracellular CB1 activity as well (Zou & Kumar, 2018). These reports identified CB1 expression within mitochondria and within intracellular compartments in non-neuronal cells, undifferentiated neuronal cells, and cultured hippocampal cells (Rozenfeld, 2011).

The ECS also consists of its endogenous ligands and the enzymes that synthesize and degrade these constituents. The ligands were identified following the identification of CB1 and CB2 when the activation of these receptors was observed with brain-derived cannabinoids (Pertwee, 2006). The two most well-known endogenous cannabinoids (i.e., endocannabinoids) are *N*-arachidonoylethanolamide (anandamide; AEA) and 2-arachidonylglycerol (2-AG) (Reggio, 2010). In addition to these endocannabinoids, CB1-interacting peptides and other arachidonic acid derivatives do exist with documented endocannabinoid-like effects (Di Marzo & De Petrocellis, 2012). As most studies focus on 2-AG and AEA, the effects of these two endocannabinoids will be discussed for the remainder of this section.

AEA and 2-AG have been identified throughout the CNS and are derived from lipid precursors. AEA is documented to be a high affinity partial agonist (a ligand that does not elicit the maximal response a full agonist does after binding to the receptor) to CB1 meanwhile having low affinity for CB2. This differs for 2-AG, which appears to be a moderate affinity, full agonist for both receptors (Reggio, 2010). However, the endocannabinoids are also known to interact with other receptors. One example is the AEA-sensitive activation of the transient receptor potential cation channel subfamily V member 1 (TRPV1) to aid in synaptic transmission and in pain regulation (Fenwick et al., 2017).

Despite the differences documented between these two endocannabinoids, their metabolism is generally similar (Figure 2). Under normal physiological conditions, endocannabinoid signaling occurs in an “on demand” fashion in response to the release of neurotransmitters from presynaptic cells (Zhou et al., 2019). Upon neurotransmitter

binding to post synaptic receptors, the enzymatic pathways to convert phospholipids (from the post synaptic cell membranes) into endocannabinoids are activated. AEA synthesis proceeds with N-arachidonylphosphatidylethanolamine-phospholipid D (NAPE-PLD) cleaving N-arachidonylphosphatidylethanolamine into AEA and phosphatidic acid. This differs from 2-AG as diacylglycerol is first created as a precursor through phospholipase C-mediated hydrolysis of membrane phospholipids in the post synaptic cell. Due to the hydrophobic nature of endocannabinoids, they likely do not diffuse freely across the synaptic cleft. The transport of these endocannabinoids across the cleft is currently still debated, but several theories have been proposed. These theories include simple diffusion driven by a concentration gradient developed by enzymatic degradation, caveolae-mediated endocytosis, fatty acid carrier proteins, and the most widely accepted theory involves an unidentified endocannabinoid transport protein system (Nicolussi & Gertsch, 2015; Chicca et al., 2012; Alger & Kim, 2011). They then bind to the presynaptic cannabinoid receptors before the endocannabinoids, AEA or 2-AG, are taken into the cell and hydrolyzed/inactivated by their respective catabolic enzymes, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Zhou et al., 2019). In addition, there is growing evidence demonstrating endocannabinoid signaling between neurons and glia (Hablitz et al., 2020; Castillo et al., 2012; Han et al., 2012; Stella, 2009). The synthetic machinery of endocannabinoids has been identified in oligodendrocytes, astrocytes, and microglia. Their functions in these cells are not entirely known, but there is evidence to suggest a role for astrocytes in indirectly modulating synaptic function due to endocannabinoid signaling (Han et al., 2012; Stella, 2009).

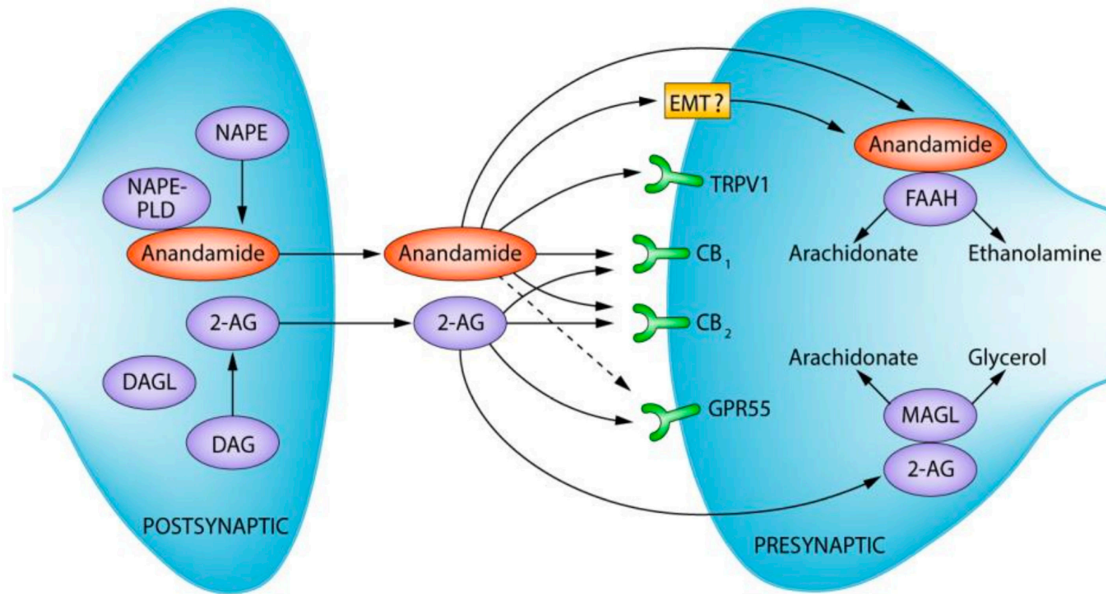


Figure 2. Schematic of the endocannabinoid system and its metabolic enzymes. This illustration is from Zhou et al. (2019) and outlines key endocannabinoids, cannabinoid receptors, and enzymes involved in endocannabinoid metabolism. DAG: Diacylglycerol; DAGL: Diacylglycerol lipase; 2-AG: 2-arachidonoylglycerol; NAPE: N-acylphosphatidylethanolamine; NAPE-PLD: N-acyl-phosphatidylethanolamine-specific phospholipase D; TRPV1: Transient receptor potential cation channel subfamily V member 1; CB1: Cannabinoid receptor type-1; CB2: Cannabinoid receptor type-2; GPR55: G protein-coupled receptor 55; MAGL: Monoacylglycerol lipase; FAAH: Fatty acid amide hydrolase; EMT: Endocannabinoid membrane transporter

1.5. Endocannabinoid signaling in the developing nervous system

Normal nervous system development is dependent on the interplay between extracellular signaling systems and gene expression during neural tissue formation. Such events that are involved in development of the CNS include appropriate patterning and regionalization of specific cells, which is dependent upon the proliferation of neural progenitor cells, specification, differentiation, and establishing the appropriate synaptic partners (Galve-Roperh et al., 2009).

It is well established in rodent models that the ECS is expressed throughout the development of the nervous system. The expression of the endocannabinoid receptors is documented to be as early as the two-cell state and one-cell state of the rodent blastocyst for CB1 and CB2, respectively (Yang et al., 1996; Maccarrone, 2009). This continues during the developmental period when the ECS contributes to the regulation of the proneurogenic and gliogenic factors involved in committing cells to their desired neuronal cell types (Harkany et al., 2007; Bertrand et al., 2002). Specifically, CB1 signaling is present during embryonic development for normal cortical development and continues to be expressed in the post-natal brain and into adulthood where it modulates adult neurogenesis (Galve-Roperh et al., 2009). Although expression of CB2 is relatively restricted to specific neuronal cell populations, there are also reports that show it plays a role in neural progenitor and NSC populations (Arevalo-Martin et al., 2007; Molina-Holgado et al., 2007).

The functions of the ECS within the developing nervous system are apparently widespread. The ECS is active within both excitatory and inhibitory neurons throughout development through excitatory projection neurons and GABAergic interneurons, respectively (Kawamura et al., 2006; Marsicano et al., 1999; Trettel et al., 2002). As mentioned above, expression of CB1 and CB2 is associated with regulating important cell fate decisions and neurogenesis (Galve-Roperh et al., 2009). There is additional evidence for roles for endocannabinoids in neural progenitor commitment, survival, and synaptic connectivity within select regions of the brain (Guzman, 2002; Aguado et al., 2006; Berghuis et al., 2007). Other processes modulated by the ECS include axonal elongation, myelination, migration, and synaptogenesis (Fernandez-Ruiz et al., 2000). As such,

alterations of normal endocannabinoid receptor signaling during nervous system development can disrupt these processes and lead to dysfunctional brain development and defective axonal growth (Watson et al., 2008).

Although most studies on the role of the ECS in CNS development to date have used rodents, there are few studies examining its presence during development of amphibians or other non-mammalian vertebrates. These organisms include *Danio rerio* (Oltrabella et al., 2017), *Taricha granules* (Soderstrom et al., 2000), *Xenopus laevis* (Cottone et al., 2003), and *Rana esculenta* (Meccariello et al., 2006). These organisms contain a relatively conserved ECS that is similarly expressed throughout the development of the brain. However, *X. laevis* is one of the few amphibians that also have the CB2 receptor identified (Beatrice et al., 2006).

1.6. Endocannabinoid signaling in response to central nervous system injury

Most of the information gathered about the potential roles of the ECS during trauma in the nervous system has been gained from studies on injury responses in regeneration-incompetent organisms, such as the rat and other mammals. Within these studies, there is evidence to show the modulation of the ECS after damage occurs in the CNS (Zhou et al., 2019). This has been studied in a wide context of injuries including contusive spinal cord injury, ischemia, and traumatic brain injury. As a result of these injuries, neuroinflammation is a main occurrence (Xiong et al., 2018). There is increasing evidence of the abundance of CB2 receptor expression on various cells of the immune system, such as neutrophils, macrophages, and lymphocytes. Furthermore, CB2 activation has also been reported concurrent with anti-inflammatory behaviours of these cells and decreased pro-inflammatory cytokine production in the area of injury (Toguri et al., 2014). Similarly,

activation of CB2 from cerebral immune cells was suggested to help limit neuroinflammation resulting from experimentally induced ischemia (Hillard, 2009). Studies that have focused on the contribution of the CB2 receptor activation using agonists additionally suggest its role in reducing leukocyte infiltration, greater recovery after experimental ischemia, and attenuating impairment after injury to the spinal cord (Zarruk et al., 2012; Walter et al., 2003; Zhang et al., 2007; Adhikary et al., 2011). These beneficial effects of CB2 activation were absent in CB2 KO mice, further indicating the important role that CB2 plays in limiting the inflammatory events after injury in the brain and spinal cord (Amenta et al., 2014).

The ECS can also be studied in the context of excitotoxicity in the CNS. This pertains to the injury or the death of neurons and other cells due to extensive exposure to excitatory neurotransmitters and the resulting influx of ions (Dong et al., 2009). In rodents, the considerable increase in CB1 expression induced by CNS injury is suggested to participate in maintaining glutamate homeostasis to reduce cell death in the CNS (Zhang et al., 2008; Ilyasov et al., 2018). The role of CB1 in regulating excitotoxicity has also been supported using CB1 KO mice that have demonstrated significant increases in cell death and a lower tolerance to excitotoxins (Marsicano et al., 2003).

After traumatic or acute spinal cord injury, the affected tissue is subjected to degeneration by secondary injury processes. Neuroprotection is the physiological response to minimize the resulting damage (Stocchetti et al., 2015). Studies performed on CB1 KO mice have indicated an increased severity of stroke, mortality, and infarct size after traumatic brain injury (Parmentier-Batteur et al., 2002). On the other hand, studies show

that the application of a CB1 agonist resulted in reduced deterioration of motor activity, cell death, and enhanced astrocytic and microglial activation (Caltana et al., 2015).

There have also been considerable changes in endocannabinoid levels and their associated enzymes that seem to be associated with neuroprotection after injury. There are numerous articles demonstrating an increase in 2-AG and AEA in rodents as early as 1 hour after receiving a contusive spinal cord injury or traumatic brain injury and this increase remains for up to 28 days (Arevalo-Martin et al., 2012; Alpár et al., 2014; Petrosino et al., 2006). In addition, an upregulation of endocannabinoid synthesizing enzymes, NAPE-phospholipase D and DAGL, and a downregulation of degradative enzymes, FAAH and MAGL, in neurons, astrocytes, and immune cells within the lesion site has also been exhibited in the injured spinal cord (Garcia-Ovejero et al., 2009; Arevalo-Martin et al., 2015). In fact, the administration of 2-AG shortly after spinal cord injury has been documented to reduce lesion expansion and also preserve the white matter located within the epicenter of the injured site (Arevalo-Martin et al., 2010).

1.7. The endocannabinoid system during CNS regeneration in regeneration-competent species

The current research on the direct role of endocannabinoids during regeneration is minimal and currently focused on invertebrates such as, the nematode and medicinal leech (*C. elegans* and *H. medicinalis*, respectively). These studies suggest the role of endocannabinoids as chemoattractants or repellents after nervous system injury. This was examined in *C. elegans* through axon regeneration assays that demonstrated the inhibitory effect of AEA on axon regeneration in motor neurons through the GPCRs NPR-19 and NPR-32 (Pastuhov et al., 2016). However, it has also been demonstrated that FAAH-1

regulates axonal regeneration in GABAergic neurons in *C. elegans* by degrading AEA (Pastuhov et al., 2012).

The main endocannabinoids and their enzymes have also been identified in the nervous system of the medicinal leech (Meriaux et al., 2011). After a lesion to the central nerve cord, there is a stark increase in both AEA and 2-AG. The authors demonstrated that AEA held a similar role as in the nematode as it did not contribute to neurite outgrowth (Meriaux et al., 2011). However, 2-AG was associated with axon extension and as a chemoattractant for microglia to the site of the injury (Arafah et al., 2013).

The role of the ECS during CNS regeneration in vertebrates is much less researched. However, studies using zebrafish, lamprey or clawed frog (*Xenopus*) models do show the regulatory role of the ECS in neural developmental processes. Such processes are required in regeneration such as axonal guidance and neurogenesis (Berghuis et al., 2007; Oudin et al., 2011; Watson et al., 2008).

1.8 Summary and main goals of thesis

The endocannabinoid system has been shown to be involved in various cellular processes that are important in the development of the nervous system and are conserved during CNS regeneration. There are also preliminary studies that have demonstrated its role in response to nerve damage in simpler nervous systems such as those of the nematode and leech. However, the studies that are performed in vertebrates are limited to organisms that demonstrate a limited capacity to regenerate from traumatic injuries in the spinal cord. These studies have demonstrated the efficacy of using pharmacological agents to study these processes and have currently shown a role for the ECS in neuroprotection, in regulating the cell fate and proliferation of NSCs, and in neurogenesis. Various glial cells

have also been cited to be responsive to CNS injury and to have the enzymes for endocannabinoid metabolism, transport, and degradation. Thus, it is likely that the ECS may play a role in the regeneration of the CNS in regeneration-competent vertebrates.

To address these gaps in knowledge, the purpose of this study was to test the hypothesis that endocannabinoid signaling plays a role in controlling the ependymoglial response and in regulating the proliferation of this NSC population after spinal cord injury in axolotls. To test this hypothesis, the following specific research aims were established: (1) To use quantitative Western blotting and indirect immunofluorescence to examine the temporal and spatial patterns of CB1 and CB2 receptor expression in the spinal cord during specific periods of caudal spinal cord regeneration. (2) To determine the gross morphological effects on tail and caudal spinal cord regeneration of constant and pulse administration of the inverse agonists, AM251 (CB1) and AM630 (CB2). (3) To correlate the spatial patterns of expression of CB1 and CB2 during caudal spinal cord regeneration with subpopulations of proliferating and differentiating neurogenic cells using selective markers (BrdU and DCX, respectively) for these subpopulations. I postulate that the endocannabinoid system acts as a modulator, regulating the response of ependymoglial cells to trauma to contribute to the creation of a permissive environment for caudal tail and spinal cord regeneration in the axolotl.

Chapter 2. Examination of Temporal and Spatial Patterns of CB1 and CB2 Receptor Expression During Caudal Tail and Spinal Cord Regeneration

2.1. Rationale

The Mexican axolotl possesses the capacity to regenerate its spinal cord and portions of its brain, making it an excellent model for studying regeneration of the nervous system (Amamoto et al., 2016). The complete transection of the axolotl spinal cord induces rapid proliferation of a population of NSCs known as ependymoglial cells. These cells are the main contributors to the regenerating spinal cord and will extend toward the wound to form the ependymal tube and bulb at the site of injury where these cells will contribute as new neurons and glial cells (Chernoff et al., 2018). Although various factors have been proposed to be responsible for regulating the regenerative response of these cells, the precise mechanisms underlying this process are yet to be fully understood.

There is evidence of the ECS active within neuroglial stem cell populations in both embryonic and adult neurogenic environments in various organisms (Zimmermann et al., 2018; Galve-Roperh et al., 2013). Various glial cells are also shown to be involved in endocannabinoid synthesis, transport, and degradation. This is observed in response to injury or inflammatory events where a sustained production of endocannabinoids from neurons and glial cells are believed to help mediate anti-inflammatory responses and neuroprotective effects (Massi et al., 2008).

Given the recorded activity of the ECS within nervous system development and in response to CNS injury, it is possible that the ECS may play a role in CNS regeneration as well. Therefore, the main aim of this chapter was to test the hypotheses that the cannabinoid receptors (CB1 and CB2) are localized in ependymoglial cells and that the expression of

these receptors will be altered in the injured caudal spinal cord injury due to tail amputation. To this end, western blotting was performed to identify potential temporal changes in both CB1 and CB2 receptor expression. Tail tissue samples harvested immediately following amputation were labelled as uninjured tissue (UINJ) and were used as the control for baseline cannabinoid receptor expression. This was then compared with regenerate tissue samples collected from specific timepoints to determine temporal expression patterns as well as timepoints allowing sufficient time for ependymal tube development. In addition, indirect immunofluorescence analyses were performed to reveal the cell and tissue-type patterns of expression as well as any potential changes in cannabinoid receptor protein localization occurring as a resulting of injury and the regenerative process.

According to previous data, it is known that the ECS is activated after damage to the brain and spinal cord to regulate neuroprotection and neuroinflammation (Stocchetti et al., 2015; Arevalo-Martin et al., 2015; Adhikary et al., 2011). The ECS is also involved in neurodevelopmental processes that are recapitulated during the regenerative process such as, axonal pathfinding and recognition of synaptic partners (Watson et al., 2008; Whalley, 2007; Berghuis et al., 2007). Furthermore, the cannabinoid receptors and ECS metabolic enzymes have been localized to various types of glia and neurons within the spinal cord (Massi, 2008; Stella, 2004), in addition to proliferating cells within neurogenic niches of the CNS (Aguado et al., 2005; Zimmermann et al., 2018; Galve-Roperh et al., 2013). Therefore, I predicted an upregulation of cannabinoid receptor signaling would occur following tail amputation, that would be localized to the ependymoglial cells within the central canal of the spinal cord. If so, these observations may suggest a role for the ECS in

regulating this stem cell population in the development of the ependymal tube in the regenerating spinal cord.

2.2. Methods

2.2.1. Animal maintenance and surgery

All experiments and procedures were approved by the Brock University Animal Care Committee (AUP no. 19-07-01). Colonies of the Mexican axolotl, *Ambystoma mexicanum*, were bred and housed in containers of dechlorinated water. Water was changed and animals were fed brine shrimp, blood worms, or fish pellets, three times a week. The animals were raised until the optimal experimental size of 2-4 cm in length. Axolotls of this length have been previously used in regeneration studies as there is a considerable amount of mature tissue, regeneration is rapid, and visualization and manipulation of the spinal cord is facilitated (Echeverri et al., 2001; Echeverri & Tanaka, 2003; Schnapp et al., 2005). When performing surgery, the animals were first anesthetized in a bath of 0.1% tricaine methane sulfonate (MS-222, Sigma-Aldrich Canada, Oakville, Ontario, Canada), pH 7.0, for 5-10 minutes. Amputation of the tail and spinal cord were performed at the mid-point between the cloaca (the reproductive and excretory orifice) and the most caudal extremity of their tail. Regenerate tissue was collected at 4 hours post amputation (hpa), and at 1, 2-, 3-, 7-, and 14-days post amputation (dpa). Approximately 10 regenerates or uninjured tissues were pooled per sample, which were then immediately flash frozen in liquid nitrogen and stored at -80°C until processed for western blotting. For immunofluorescence staining, sections were immediately fixed in 4% paraformaldehyde prior to processing. (see sections 2.2.2. and 2.2.3 for further details). All regenerate tissues were collected from animals that had not been subjected to more than one amputation.

2.2.2. Western blotting

Frozen tissue samples (n=3 for each timepoint where each n is comprised of 10 regenerates) were prepared by homogenization in lysis buffer, followed by centrifugation for 30 minutes at 4°C. Total protein concentrations were measured using the BCA protein assay (Pierce, Rockford, IL, USA) and 20 μg of total protein per sample were combined with the Laemlli loading buffer. These samples were loaded and run on a 12% polyacrylamide resolving gel, and a 4% stacking gel for approximately 2.5 hours at 80V. A wet western blot transfer was then performed onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Oakville, Ontario, Canada) followed by Ponceau S (Sigma Aldrich, Canada) total protein staining for 5 minutes. The membranes were briefly rinsed in 4% acetic acid and MilliQ water and were left to dry prior to imaging the total protein stain. All images for western blots were obtained using a ChemiDoc MP imaging system (Bio-Rad, Canada) and imaging was followed by reactivation in methanol and washed with 0.1%Tween-20/TBS. The membranes were then blocked in 3% non-fat skim milk powder/0.1% Tween-20/PBS for 1 hour while shaking at room temperature. Overnight incubations in primary antibodies were then performed at 4°C using 1:1000 ACR-1 (Alomone labs, Jerusalem, Israel), and 1:1000 ACR-2 (Alomone labs, Jerusalem, Israel). The following morning, the membranes were washed in 0.1%Tween-20/TBS and incubated with a secondary antibody (1:15,000) Alexa Fluor 680 goat anti-rabbit IgG (Invitrogen), or Alexa Fluor 680 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) depending on the primary antibody used, for 45 minutes at room temperature. Three biological and technical replicates were performed for each condition.

2.2.3. Immunofluorescence staining

Tail tissues were fixed for 24 hours in 4% paraformaldehyde at 4°C immediately after primary tail amputation and at subsequent time points. This was followed by cryoprotection with a stepwise series of 30-minute sucrose washes (5% sucrose to 30% sucrose) at room temperature on a rotating platform. The final wash of 30% sucrose was performed overnight at 4°C and then the tissues were stored in a 2:1 solution of 30% sucrose:OCT (optimal cutting temperature compound, VWR, Pennsylvania, USA) at -20°C. The tissues were cut into 15μm sections with a Cryomicrotome FSE (Thermoscientific), then collected on Superfrost Plus (VWR, Pennsylvania, USA) slides. Tissues used for staining underwent washes using PBS and PBS-Triton. The slides were then blocked using 5% normal goat serum (Sigma Aldrich, Canada) in PBS-Triton. Incubations in primary antibodies took place overnight in an airtight container at 4°C. The concentrations of primary antibodies were as follows: 1:100 ACR-1 (Alomone labs, Jerusalem, Israel), 1:100 ACR-2 (Alomone labs, Jerusalem, Israel) antibodies, and 1:50 GFAP (DSHB, Iowa, USA). Negative controls were also performed by omitting the primary antibody. The following morning, the samples were washed using PBS-Triton and prepared for incubation in the secondary antibody at room temperature for 1 hour. A 1:200 dilution was used for either the Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) or the Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA). This was followed with PBS-Triton and PBS washes and application of Fluoroshield with DAPI (Sigma Aldrich, Canada) and a coverslip.

2.2.4. Immunofluorescence imaging

The stained slides were then imaged using a Zeiss Axio Observer.Z1 confocal microscope (Zeiss Canada, North York, Ontario, Canada) equipped with ApoTome.2 optical sectioning, 63x oil objective, and Hamamatsu ORCA-Flash4.0 V2 digital camera (Hammamatsu, Hammamatsu City, Shizuoka, Japan). An X-Cite 120 LED light source was used as the fluorescent light source. Z-stacks were performed on all samples with slices in 0.24μm intervals. All confocal images were processed with maximum intensity projection from an extended depth of focus on the Zeiss Zen 2 microscopy software. The Alexa Fluor 488 signal was captured using set excitation and emission wavelengths of 488nm and 509nm through excitation and emission wavelength filter sets of 450-490nm and 500-550nm. The Alexa Fluor 594 signal was captured using set excitation and emission wavelengths of 587nm and 610nm through excitation and emission wavelength filter sets of 540-602nm and 590-660nm. Signal from DAPI was captured using set excitation and emission wavelengths of 353nm and 465nm through excitations and emission wavelength filter sets of 335-383nm and 420-470nm. All representative images were taken using the exact same exposure times and settings.

2.2.5. Statistical analyses

Data were analyzed and illustrated using Graphpad Prism 8 for Windows (La Jolla, CA, USA). A One-Way ANOVA paired with a Tukey's *post-hoc* test was also used to determine statistical significance during different stages of regeneration. P-values less than 0.05 were considered statistically significant for all analyses.

2.3. Results

2.3.1. CB1 expression increases at 4 hpa and is sustained to 14 dpa

My first aim was to discover if CB1 expression is altered during the course of regeneration after spinal cord and tail amputation. However, there are few CB1 antibodies available and there are no known antibodies available either commercially or in other laboratories raised against the CB1 sequence in the axolotl. The antibody employed in this experiment was provided by Alomone Labs and is specific to the CB1 sequence in the rat, *R. norvegicus*. Therefore, the CB1 sequences of the axolotl and the rat were aligned to identify the conservation of these sequences, specifically within the antigenic epitope (Figure 3). The amino acid sequence of the axolotl CB1 is 81.89% homologous to the rat sequence and the antigenic determinant for the antibody lies within the extracellular N-terminus corresponding to amino acid residues 84-99 of rat CB1. Although the epitope indicated in the red box does not present full conservation (represented by the asterisk above each amino acid residue), there are substitutions with strongly similar properties (represented by the colon) (Figure 3). Based on this analysis, this antibody was employed as it was likely to bind to the axolotl CB1.

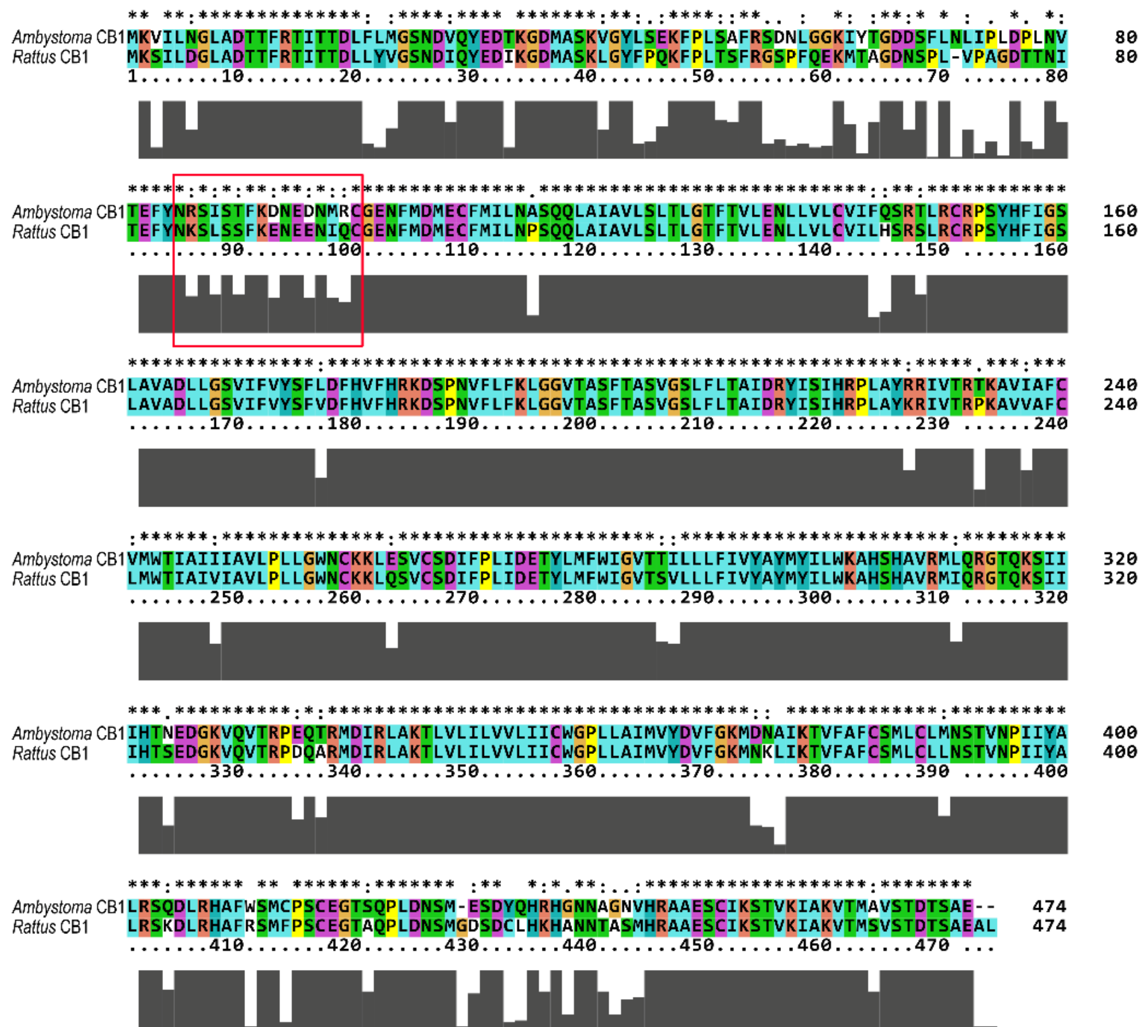


Figure 3. CB1 protein alignment between *A. mexicanum* and *R. norvegicus*. There is 81.89% homology across the entire CB1 sequence between these two organisms. The red box indicates the epitope indicated from the rat CB1 antibody provided from Alomone labs. Symbols above each amino acid indicate level of conservation, whereas each colour represents the property of each residue. Symbol Legend: Asterisk (fully conserved residue), Colon (residues with strongly similar properties and >0.5 on Gonnet PAM 250 matrix), Period (residues with weakly similar properties and <0.5 on Gonnet PAM 250 matrix). Colour Legend: Blue (hydrophobic residue), Red (positive charge), Magenta (negative charge), Green (Polar), Orange (glycines), Yellow (proline), Cyan (Aromatic), White (unconserved).

To examine potential temporal differences in CB1 expression during regeneration, western blotting was performed on regenerating tail and spinal cord tissue at specific time points post amputation. Ratiometric analyses of CB1 expression during the early stages of regeneration shows a significant increase in CB1 expression following amputation (Figure 4A). After only 4 hours, a significant increase in protein expression was observed in the 120 kDa band and was maintained for 3 days ($F_{(4, 40)} = 5.994$, $P = 0.0007$). According to the Tukey *post-hoc* analysis, there was significant 4-fold upregulation 4 hpa ($P = 0.017$), 1 dpa ($P = 0.0433$), 2 dpa ($P = 0.0013$), and 3 dpa ($P = 0.0013$) compared to uninjured tissue (UINJ; time 0). Ratiometric analyses also identified a significant increase in CB1 expression 7 and 14 dpa ($F_{(2, 24)} = 15.97$, $P < 0.0001$) (Figure 4B). Specifically, CB1 expression increased 7 dpa ($P = 0.0369$) and 14 dpa ($P < 0.0001$). CB1 expression in regenerates from 14 dpa also appeared to be significantly higher in 14 dpa compared to 7 dpa ($P < 0.0161$). A preadsorption control with the antigenic peptide was performed on tail and spinal cord tissue (Figure 4C). The rat CB1 control peptide successfully blocked CB1 antibody binding. This suggests that this antibody recognizes and binds to a similar antigenic determinant of the axolotl CB1. The CB2 control peptide was also used in the place of the CB1 peptide to exclude the possibility of a cross reaction between similar determinant on CB2 and the CB1 proteins. The 120kDa band remained in the presence of the CB2 peptide-preadsorbed CB2 antibody, strongly arguing against cross-reactivity. These data indicate the significant upregulation in CB1 expression within the initial timepoints of regeneration that appears to diminish somewhat at 7 and 14 dpa. However, at 7 and 14 dpa, CB1 expression remains significantly higher than within the UINJ tissue.

This 120kDa band is considerably larger than the expected molecular weight of 53kDa based on the amino acid sequence of the axolotl CB1; however, this band was successfully blocked by the control peptide in testing for antibody specificity. Furthermore, results from experiments (see Chapter 4) with an inverse agonist specific to the rat CB1 receptor show a significant inhibition of tail regeneration in the axolotl suggesting conservation in the receptors at that binding site. There is evidence in the literature to justify this high molecular weight band. The cannabinoid receptors are particularly likely to be phosphorylated or glycosylated given the specific amino acid motifs on the N terminus of these receptors (De Jesus et al., 2006; Samson et al., 2003). In addition, dimerization within the Class A GPCR family is well documented and is suggested to contribute to important functions (Terrillon & Bouvier, 2004). Svetlov et al. (2009) also reported the generation of 120 kDa and >260 kDa complexes for CB1 in the rat cortex and hippocampus after traumatic head injury, suggesting a potential role for this high molecular weight putative CB1 complex during nervous system damage. This 120 kDa complex was also found by Wager-Miller et al. (2002), who showed its insensitivity to the reducing agents DTT, NES, and iodoacetamide. Most studies on CB1 dimer complexes suggest homodimerization, but there is pharmacological evidence to suggest that dimerization with dopamine, serotonin, and opioid receptors is also possible (Wager-Miller et al., 2002). Although the data presented here cannot distinguish between homo or heterodimerization, these data do suggest that dimerization and glycosylation may be factors that contribute to the 120 kDa band for CB1.

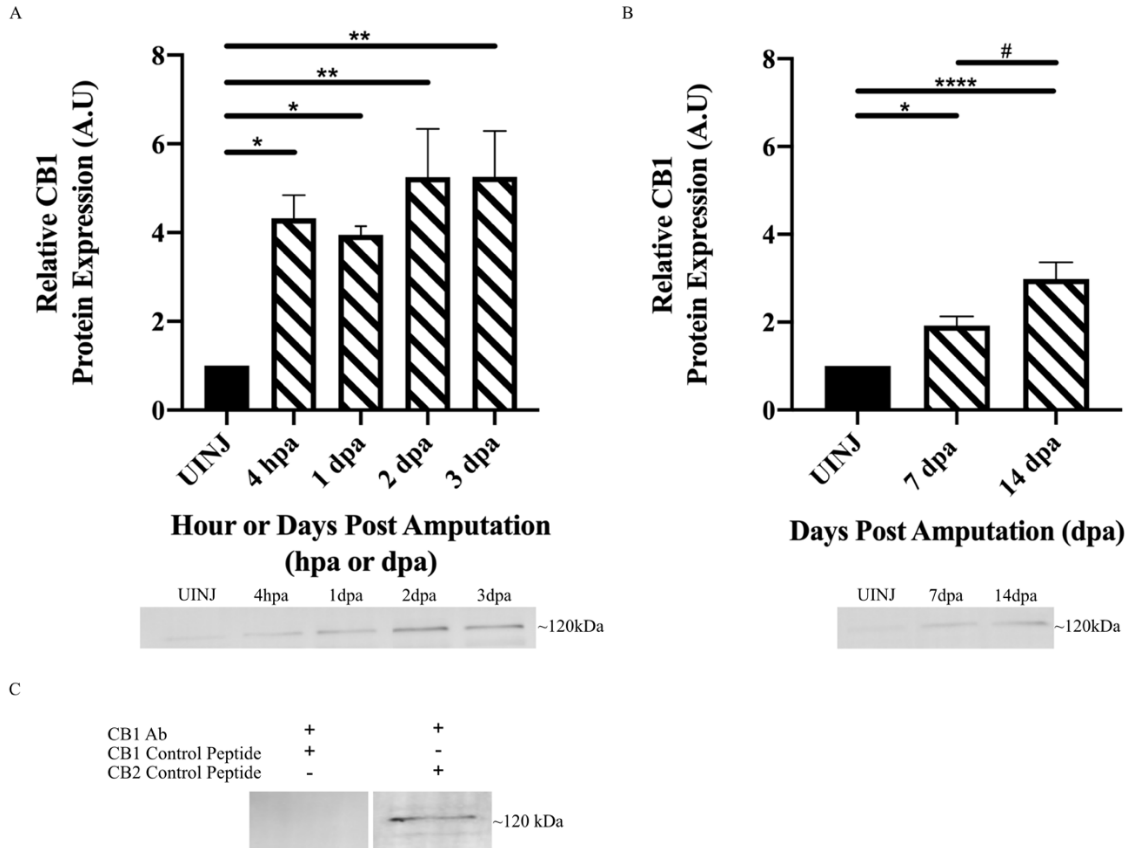


Figure 4. CB1 protein expression significantly increases following tail and spinal cord amputation. A and **B)** Ratiometric analyses of CB1 protein expression within caudal tail regenerate tissue after tail and spinal cord amputation. * or # ($P < 0.05$), ** ($P < 0.01$), **** ($P < 0.001$). N=3 for biological and technical replicates. **C)** Preadsorption protocol for the CB1 antibody was performed using both the CB1 and CB2 peptides provided from Alomone labs. Western blotting data are separated into two graphs as these tissues were collected from animals of different breeding cohorts.

2.3.2. Elevation of CB2 expression is restricted to 7 and 14 dpa

I next aimed to discover if there was a difference in CB2 expression during the same time course of tail regeneration. Similar to CB1, there are not many commercial antibodies available for CB2 and there is no antibody that is developed specifically for the axolotl. A sequence alignment was performed between the rat and axolotl CB2 sequences

and revealed 48.23% homology between them (Figure 5). The CB2 antibody from Alomone Labs was employed in the current study and is directed to the 3rd intracellular loop corresponding to amino acid residues 228-242 of the rat CB2, illustrated by the red box. Unlike the sequence alignment for CB1, this fails to show fully conserved residues in the antigenic region but does have some residues with strongly similar properties.

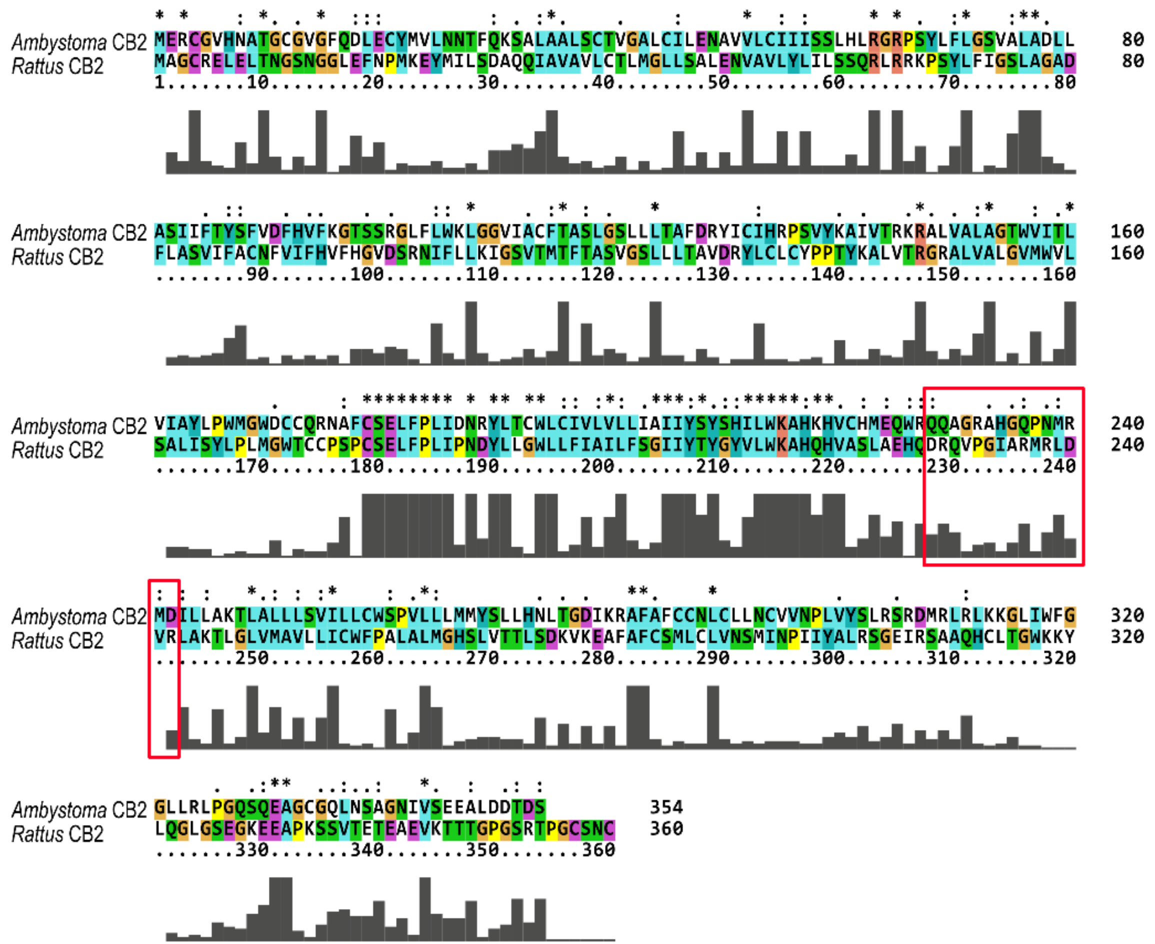


Figure 5. CB2 protein alignment between *A. mexicanum* and *R. norvegicus*. There is 48.23% homology across the entire CB2 sequence of these two organisms. The red box indicates the epitope indicated from the rat CB2 antibody provided from Alomone labs. Symbols above each amino acid indicate level of conservation, whereas each colour represents the property of each residue. Symbol Legend: Asterisk (fully conserved residue), Colon (residues with strongly similar properties and >0.5 on Gonnet PAM 250 matrix), Period (residues with weakly similar properties and <0.5 on Gonnet PAM 250 matrix). Colour Legend: Blue (hydrophobic residue), Red (positive charge), Magenta (negative charge), Green (Polar), Orange (glycines), Yellow (proline), Cyan (Aromatic), White (unconserved).

Western blotting was again employed to determine, quantitively, the temporal changes in CB2 expression following spinal cord injury. Ratiometric analyses on CB2 expression during the early stages of regeneration revealed an elevation in CB2 expression between UINJ and 3 dpa ($F_{(4, 40)} = 2.779$, $P = 0.0397$ (Figure 6A). However, the *post-hoc* analysis demonstrated that there are no significant differences between UINJ and any regenerate tissue during the first 3 dpa. CB2 expression also appeared to be significantly different at 7 and 14 dpa ($F_{(2, 24)} = 10.84$, $P = 0.0004$) (Figure 6B). My results show a significant 2-fold increase in CB2 expression at 7 dpa ($P = 0.002$) and 14 dpa ($P = 0.001$). A preadsorption control was also performed using the control peptide (Figure 6C). Its purpose was to determine whether the CB2 antibody used in these studies would specifically bind to what we believe is CB2 in the axolotl. The CB2 control peptide successfully blocked CB2 antibody binding suggesting that this antibody is binding to the CB2 receptor in the axolotl. The CB1 control peptide was also used in place of the CB2 peptide to address the possibility of cross reactions between the CB2 antibody and the CB1 receptor epitope. Again, the 46 kDa band was still present when the antibody was preabsorbed with the CB1 peptide, suggesting that no cross reactivity occurred. These data thus indicate an upregulation in CB2 expression during tail regeneration. However, only at 7 and 14 dpa was this found to be significantly different compared to uninjured control tail and caudal spinal cord tissue.

The current study identified bands for CB2 measured at 46 kDa and 55 kDa, which are higher than the predicted molecular weight of 39 kDa according to its amino acid sequence. The CB2 receptor in rats and mice has previously been documented to produce doublet bands with similar molecular weights using a variety of antibodies (Fukuda et al.,

2014; Álvaro-Bartolomé et al., 2013, Merriam et al., 2008; Walczak et al., 2006). There are multiple possible theories for the observed doublets; however, it is likely that these may be from potential isoforms of CB2 or posttranslational modifications such as glycosylation (Liu et al., 2008; Jordan & Xi, 2018; Filppula et al., 2004). Furthermore, a majority of the current studies in vertebrates were conducted on mammalian models and no data about CB2 has been published from urodeles. Indeed, antibody specificity for the CB2 receptor has been an issue for debate within the literature (Ashton, 2012; Atwood & Mackie, 2010), but many of these critiques were not associated with the antibody used in the current study. In fact, the CB2 antibody used here was previously employed in recent studies as it was evidenced to have greater CB2 specificity compared to other commercially available antibodies (Zhang et al., 2014; Zhang et al., 2017).

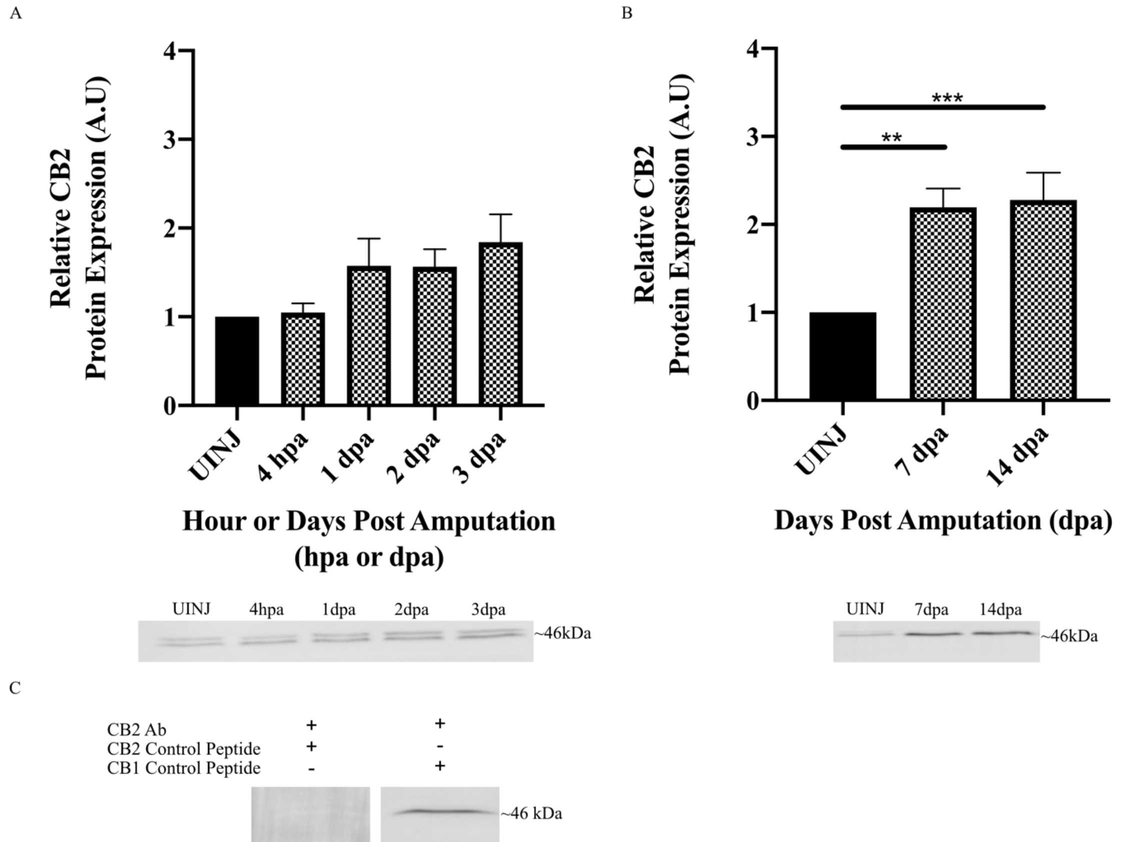


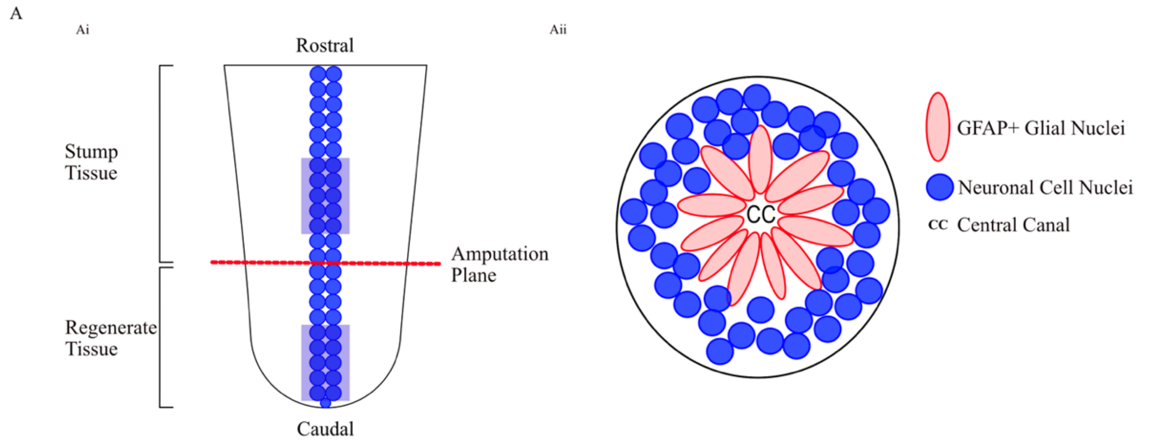
Figure 6. CB2 protein expression significantly increases following tail and spinal cord amputation. A) and B) Ratiometric analyses of CB2 protein expression within caudal tail regenerate tissue after tail and spinal cord amputation. ** ($P < 0.01$), *** ($P < 0.001$). N=3 for biological and technical replicates. C) Preadsorption controls for CB2 antibody. Preadsorption protocol was performed using both the CB1 and CB2 peptides provided from Alomone labs. Western blotting data are separated into two graphs as these tissues were collected form animals of different breeding cohorts.

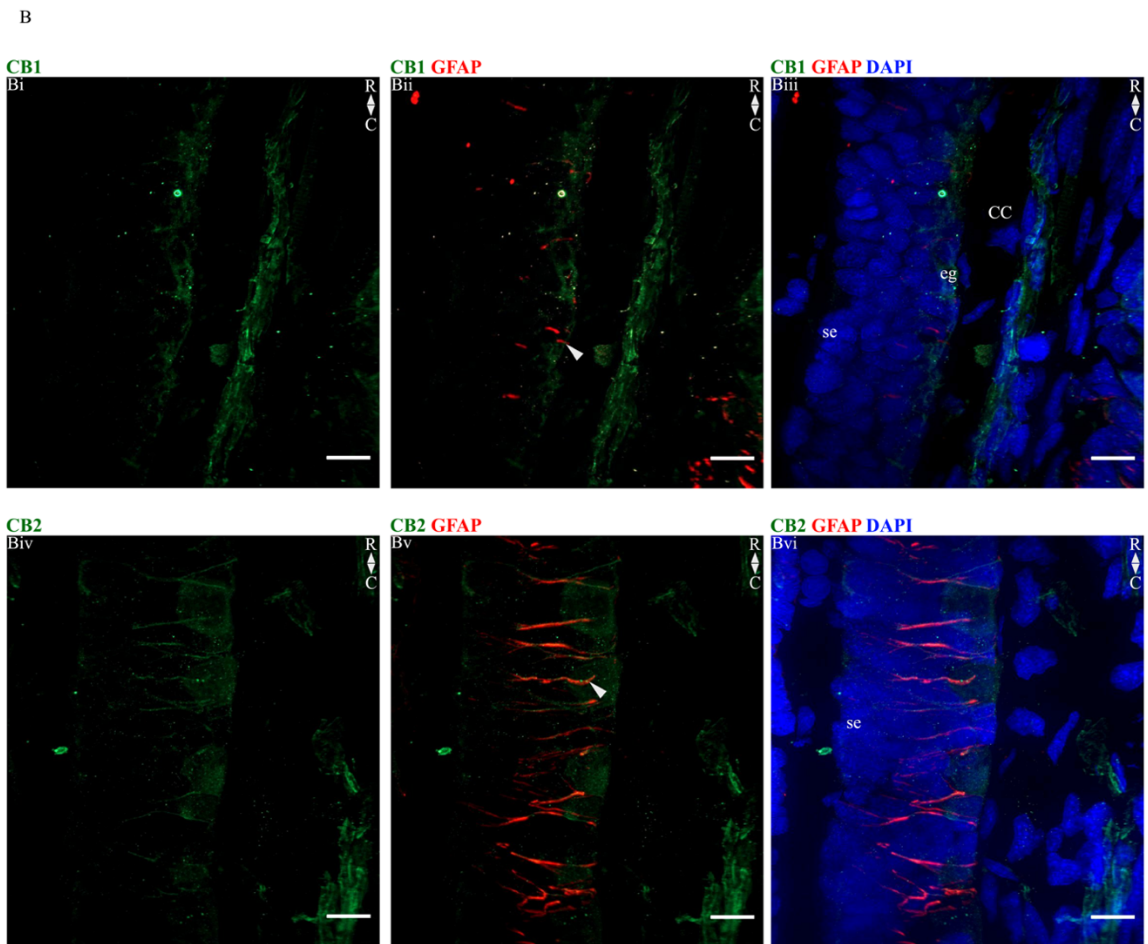
2.3.3. CB1 expression is restricted to ependymoglial cells while CB2 expression is found within the subependymal region of the spinal cord

After identifying stage-specific increases in the expression of both CB1 and CB2, I was interested in determining the precise tissue and cellular localization of these CB1 and CB2 receptors. Immunofluorescence analyses and imaging were performed on UINJ and 7 dpa regenerate sections. The images were obtained rostral to the original amputation plane

to determine whether the measured upregulation of these receptors was occurring in remaining tail stump or in newly regenerated tissues.

UINJ tissue was used for initial observations of the cellular domains of these receptors. Within this tissue, occasional overlap between the localization of either CB1 and GFAP (an ependymoglial marker) or CB2 and GFAP was observed (white arrows; Figure 7 Bii, Bv, and Cii). This suggests that both endocannabinoid receptors can be expressed within the population of ependymoglial cells. However, for the most part, the expression of the two receptors appears to be non-overlapping. The CB1 receptor appeared to be enriched in the GFAP+ ependymoglial cells that line the central canal of the spinal cord (Figure 7 Biii and Ciii). CB2 was also found in the spinal cord but was far more abundant within subependymal cells peripheral to the ependymoglial layer (Figure 7 Bvi and Cvi).


A
Ai
Aii
Rostral
Stump
Tissue
Amputation
Plane
Regenerate
Tissue
Caudal
CC
GFAP+ Glial Nuclei
Neuronal Cell Nuclei
cc Central Canal


B
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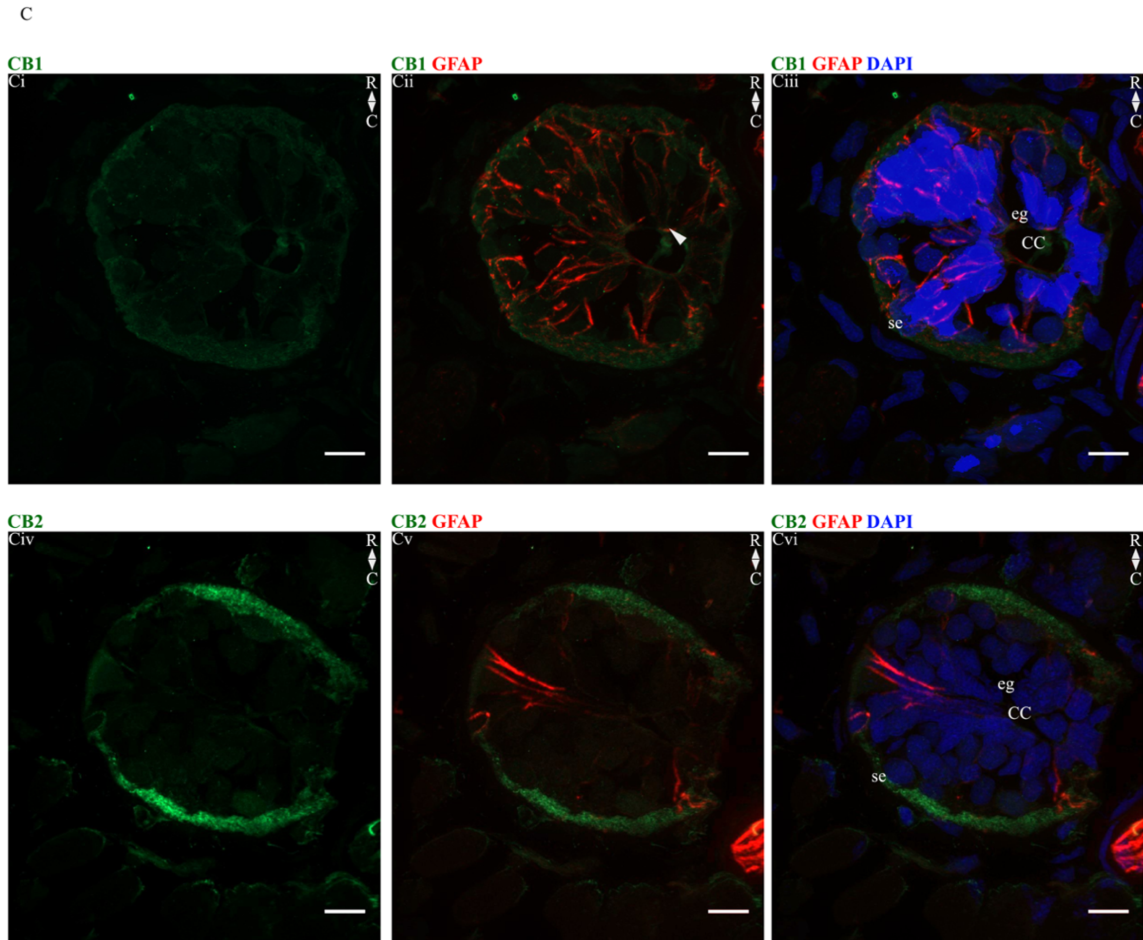


Figure 7. Tissue-specific expression of cannabinoid receptors (CB1 and CB2) in uninjured spinal cord tissue. A) Schematic diagrams of a regenerating axolotl tail. i) Sagittal section of the regenerating axolotl tail. Red line marks the amputation plane (AP), which separates the stump tissue (rostral from the amputation plane) from the regenerate tissue (caudal from the amputation plane). Blue circles represent the ependymoglial cells of the spinal cord. Purple boxes indicate approximate area of immunofluorescence images. ii) Cross section of the regenerating axolotl tail. Blue circles represent neuronal cell nuclei, and the red elliptical shapes represent GFAP+ ependymoglial cell nuclei, which are found along the central canal (CC) of the spinal cord. **B)** Sagittal sections displaying the location of CB1 or CB2, and GFAP protein expression in uninjured tissue. **C)** Cross sections displaying the location of CB1 or CB2, and GFAP protein expression in uninjured tissue. White arrowheads indicate area of CB1 or CB2 co-localization with GFAP. Scale

bars for all images set at 50 μm. cc: Central canal; eg: ependymoglia; se: subependymal cells. N=3 for biological and N=3 or 4 technical replicates.

As mentioned previously, ependymoglial cells represent a stem cell population critical to the supply of new neurons and glia within the regenerating axolotl tail and spinal cord (Chernoff et al., 2018). Given the critical role of GFAP+ cells within spinal cord regeneration in the axolotl, it is possible that cannabinoid signaling may have a function in regulating the numbers of GFAP+ ependymoglial cells after injury.

GFAP has been shown in multiple reports to be downregulated in the axolotl spinal cord in response to trauma (O'Hara et al., 1992; Sabin et al., 2019). Thus, the next objective was to confirm, using quantitative Western blotting, that this downregulation of GFAP expression is reproducible. Similar to previous findings, we found GFAP expression was downregulated after amputation ($F_{(3, 432)} = 25.97$, $P < 0.0001$). A significant downregulation in GFAP was observed during all timepoints when compared to the regenerate tissue ($P < 0.001$) (Figure 8).

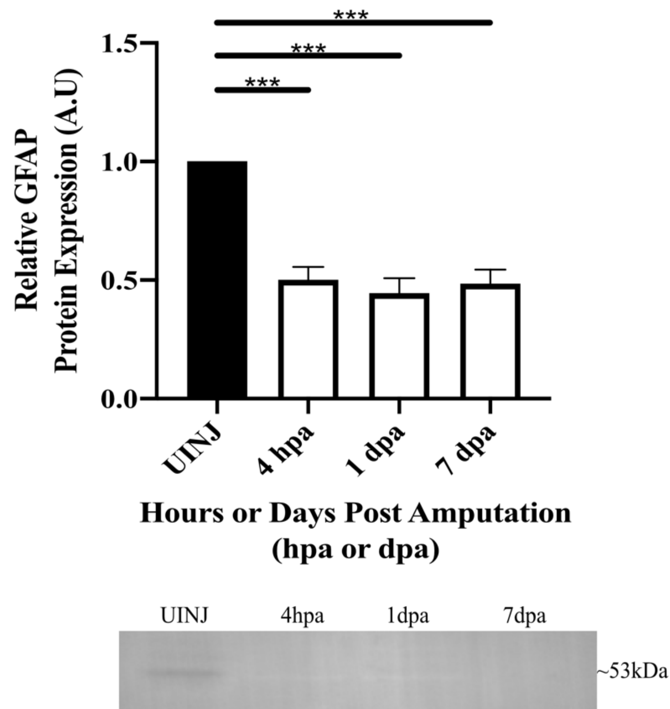
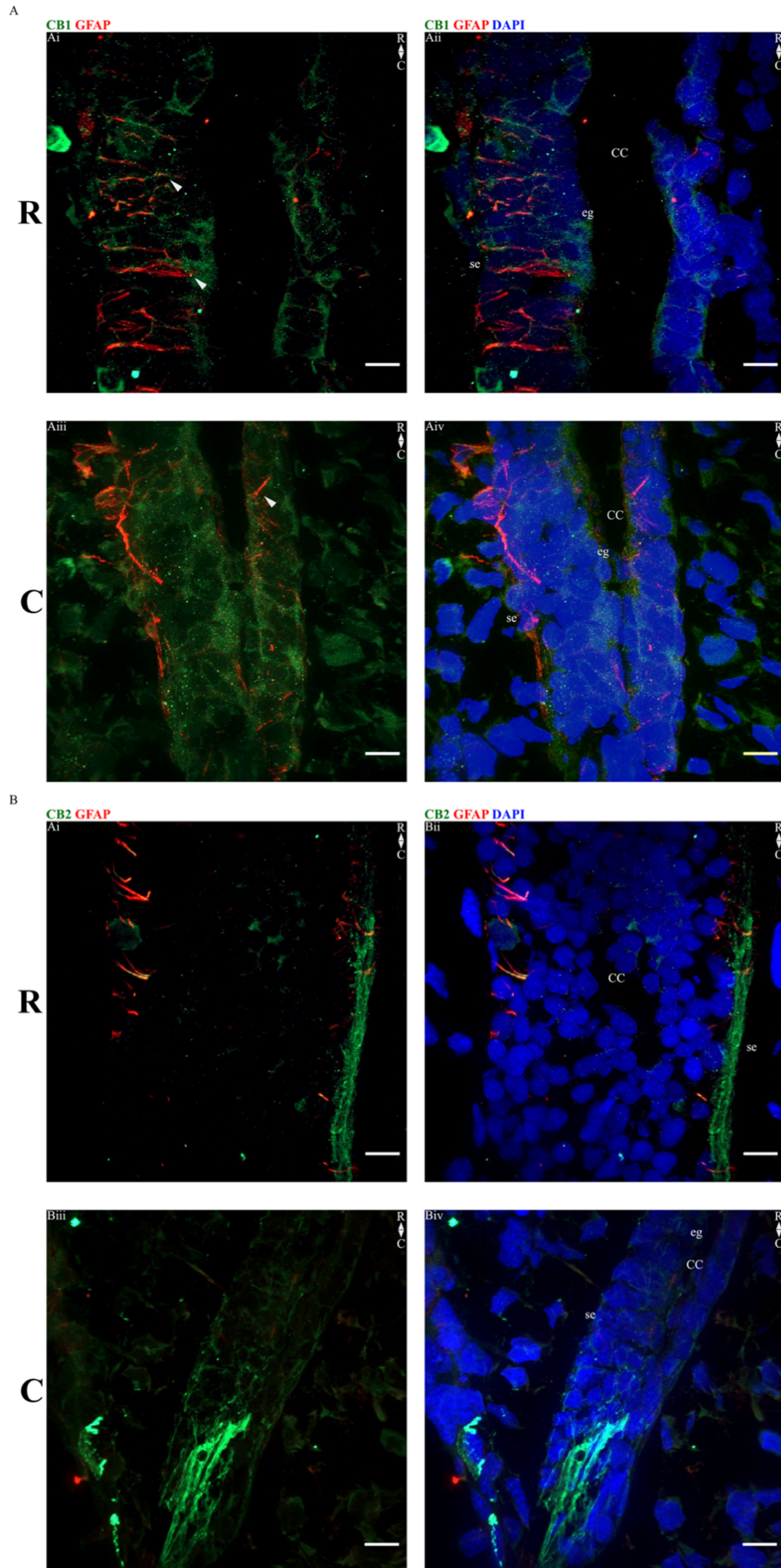


Figure 8. Western blot data showing the downregulation of GFAP during tail and spinal cord regeneration. Ratiometric analysis of GFAP protein expression showing downregulation of GFAP over the course of regeneration. *** ($P < 0.001$) and N=3 biological and technical replicates for each condition.

Our previous western blot data demonstrated an upregulation of CB1 and CB2 at 7 dpa (Figures 4B and 6B). Therefore, the next aim was to determine the precise localization of CB1 and CB2 expression at 7 dpa. Specifically, I was interested in which cells in the spinal cord demonstrated the upregulation in cannabinoid receptors and whether GFAP was downregulated in ependymoglia according to previous data. Through immunofluorescence imaging, a general increase in the abundance of each receptor at 7 dpa was observed within the caudal sections of the spinal cord. The CB1 receptor was expressed almost exclusively in ependymoglial cells where it co-localized with GFAP (Figure 9Ai and Aiii). This differed from CB2 receptor, which displayed less overlap with

GFAP and was mainly found in the subependymal (GFAP-) region. In both Figures 9A and 9B, a qualitative decrease in GFAP expression can be identified in the caudal sections when compared to the rostral sections. Negative controls omitting the primary antibody were performed and did not show any fluorescence signaling in these samples.


A
CB1 GFAP
Ai
R
C
R
CB1 GFAP DAPI
Aii
cc
eg
se
Aiii
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Aiv
cc
eg
se
B
CB2 GFAP
Ai
R
CB2 GFAP DAPI
Bii
cc
se
Biii
C
Biv
eg
cc
se

Figure 9. Tissue-specific expression of cannabinoid receptors (CB1 and CB2) in regenerating spinal cord tissue 7 days following amputation. A) Sagittal sections displaying the location of CB1 and GFAP protein expression in 7-day regenerate tissue. Images in the R row indicate sections that were taken rostral to the amputation plane (stump tissue), while images in the C row indicate sections that were taken caudal to the amputation plane (regenerate tissue). **B)** Sagittal sections displaying the location of CB2 and GFAP protein expression in 7-day regenerate tissue. Images in the R row indicate sections that were taken rostral to the amputation plane (stump tissue), while images in the C row indicate sections that were taken caudal to the amputation plane (regenerate tissue). White arrowheads indicate areas of CB1 or CB2 co-localization with GFAP. Scale bars for all images set at 50 μm. cc: Central canal; eg: ependymoglia; se: subependymal cells. N=3 for biological and N=3 or 4 technical replicates.

2.4. Discussion

2.4.1. Summary of main findings

The current study presents the first data showing modulation of the ECS in response to tail and spinal cord injury in a regeneration-competent vertebrate, the axolotl. These data demonstrate that both CB1 and CB2 receptor expression are upregulated during tail and spinal cord regeneration at various stages after resection of the tail. Moreover, the expression of both receptors occurs rostral to the site of injury as well as caudally in the regenerate tissue. The current study also presented evidence that these receptors were expressed primarily within the ependymoglial cells (CB1) and subependymal cells (CB1 and CB2). The ependymoglial cells have been previously described as a primary NSC population bordering the central canal of the spinal cord. These cells are the main contributors to spinal cord regeneration as injury induces their rapid proliferation to allow

the development of the ependymal tube and bulb and eventually to the production of new neurons and glial cells (Chernoff et al., 2018). Radially peripheral to the ependymoglial cells is the subependymal layer (Hui et al., 2010). The subependymal cells consist of cells whose cell bodies reside outside the central canal and provide an intermediate zone between migrating neuroblasts and ventricular layers (Zamore, 1978; Ashner, 2010). Within this subependymal region there are immature, actively dividing cells that form various newly generated neurons, oligodendrocytes, and astrocytes (Zamore, 1978; Hui et al., 2010).

2.4.2. Physiological relevance of injury-induced cannabinoid receptor upregulation

As mentioned previously, the role of glia in response to injury between CNS regeneration-incompetent and competent organisms differ drastically. Recently, however, the contributions of the ECS in glia functionality has become a promising avenue for biomedical applications as various glia are now known to display the enzymes for endocannabinoid synthesis, transport, and degradation (Luongo et al., 2010; Stella, 2004; Scheller & Kirchhof, 2016). Thus, it is important to examine the possibility of the involvement of the ECS in the pro-regenerative response of ependymoglial cells present in the axolotl but absent within regeneration-incompetent organisms.

Within the spinal cord, there is evidence to suggest the ECS is expressed and may play a role in regulating the behaviour of ependymal cells within regeneration-incompetent mammals. Paniagua-Torija et al. (2015) identified abundant CB1 expression within the GFAP+ astrocytic domains within the ependymal regions of human spinal cords and forming part of the astrogliotic response associated with spinal cord closure. They

identified strong CB1 expression on reactive astrocytes within pathological samples (Paniagua-Torija et al., 2015). CB1 was also found within reactive astrocytes in pathological spinal cord samples suggesting that CB1 may be modulated according to changes in metabolism, protection, or inflammation (Carracedo et al., 2004; Molina-Holgado et al., 2003).

A subpopulation of ependymal cells that exhibit a high expression of CB1 ($CB1^{HIGH}$ cells) residing around the central canal of the spinal cord have been identified in humans, rats, and mice (Paniagua-Torija et al., 2015; Garcia-Ovejero et al., 2012). This cell population expresses stem cell/precursor markers such as, vimentin, nestin, Sox2, Sox9, and GLAST (Garcia-Ovejero et al., 2012). Examining this cell population in rats, Garcia-Ovejero et al. (2012) discovered that these cells exhibit quiescence in the intact adult spinal cord. However, these cells enter the cell cycle (expressing the proliferation marker Ki67) in response to a spinal cord contusion and a small proportion of them were observed to have proliferative properties postnatally as well (Garcia-Ovejero et al., 2012).

Similarly, there are numerous reports of CB2 within the mammalian spinal cord (Adhikary et al., 2011; Arevalo-Martin et al., 2012; Baty et al., 2008) and within specific glial populations such as, microglia (Stella, 2009; Maresz et al., 2005). It is possible that the CB2 expression I observed may be from microglia within the spinal cord as previous studies have shown CB2 expression in various immune cells and the endocannabinoid-induced activity of these cells during neuropathology (Tanaka et al., 2020; Maresz et al., 2005). This could be an interesting finding as it is well known in nervous system regeneration models (such as the zebrafish) that microglia have a role in modulating development and plasticity during nervous system regeneration (Var & Byrd-Jacobs,

2020). In addition, interactions between microglia and NSCs in neurogenic niches has been shown in the past (Sirerol-Piquer et al., 2019) and microglia have been cited to release high levels of endocannabinoids to affect nearby cells (Garcia-Ovejero et al., 2013).

There are various reports supporting the anti-hyperalgesic (increased sensitivity of pain) or anti-allodynic (pain resulting from a stimulus that would not usually cause pain) effects of anandamide activation of CB1 signaling, which might explain the initial upregulation of CB1 observed after tail amputations in the current study (Hama & Sagen, 2011; Farquhar-Smith et al., 2000; Munawar et al., 2017; Rahn & Hohmann, 2009). Similar results were observed as activation of both cannabinoid receptors through WIN55,212-2 (an agonist for both CB1 and CB2) in the Wistar rat led to the reversal of neuropathic pain in this model (Bridges et al., 2001). In addition, Bridges et al. (2001) found that these effects were reversed after application of a CB1-selective antagonist, but not CB2-selective antagonist, suggesting an important role for CB1 activation in the mediation of allodynia and hyperalgesia. This is further supported by previous studies by Rao et al., (2009) who identified a consistent upregulation in the 2-AG synthesizing enzyme, DAGLB, after amputation of the axolotl limb. Although this focused on limb amputation, these authors attribute this finding to the effect of 2-AG on axonal growth and pain control (Rao et al., 2009).

However, my current study demonstrated that CB1 upregulation was maintained for at least 14 dpa, which may be due to its involvement in other processes. It is reasonable to suggest that CB1 upregulation has been associated with neuroprotective roles in various models in attenuating secondary damage or promoting functional recovery (Price et al., 2009; Kendall & Yudowski, 2016; Saito et al., 2012). The CB1 receptor is known to be

expressed in the intact spinal cords in astrocytes, oligodendrocytes, microglia, and ependymal cells and rodents (Salio et al., 2002; Hegyi et al., 2009; Garcia-Ovejero et al., 2009; Garcia-Ovejero et a., 2012). Other studies identify the regional expression CB1 within neurons and glia near the epicenter of a spinal cord lesion (Arevalo-Martin et al., 2015). This is similar to the findings of this thesis that suggests the upregulation of CB1 and CB2 within the ependymoglial and subependymal cells of the caudal spinal cord.

The upregulation of CB1 is observed in various neurodevelopmental processes that are also required during regeneration. Such processes include axonal guidance, modulation of synaptic plasticity, and cell proliferation in vertebrates (Aguado et al., 2006; Arafah et al., 2013). There is evidence indicating a role for the ECS within these processes in vertebrates that do display spinal cord regeneration; however, there are yet to be any findings within the axolotl. Within the lamprey, Kyriakatos & Manira (2007) demonstrate a role for endocannabinoids in inducing long-term synaptic plasticity in the spinal cord and reconfiguration of the locomotor networks after spinal cord injury. Similarly, it was found within *Xenopus,* that endocannabinoids act as axonal guidance cues as well as playing a role in regulating synaptogenesis (Berghuis et al., 2007). In addition, a role for the ECS has also been displayed in the zebrafish where previous reports suggest that CB1 aids in regulating axonal growth and pathfinding of forebrain and hindbrain neurons during early neurodevelopment (Zuccarini et al., 2019; Watson et al., 2008). In fact, the experimental knock down of CB1 signaling by morpholino injection impairs axonal growth and fasciculation, neurogenesis, and neural progenitor proliferation and migration in the zebrafish (Oudin et al., 2011; Watson et al., 2008). Given that these are events in nervous system development that are recapitulated during regeneration, it is possible that these are

processes that are modulated by endocannabinoid signaling during the regenerating spinal cord in the axolotl as well.

Chapter 3. Examining the Effects on Tail and Caudal Spinal Cord Regeneration of Constant and Pulse Administration of the Inverse Cannabinoid Receptor Agonists, AM251 (CB1) and AM630 (CB2)

3.1. Rationale

In the previous chapter, I have identified an injury-induced upregulation of CB1 and CB2 protein expression. This upregulation was identified within particular cellular domains in the regenerating spinal cord such as the ependymoglial cells and the subependymal cells respectively. The current chapter now investigates whether these receptors are functional during regeneration and to test the hypothesis that cannabinoid receptor signaling is required for normal tail and spinal cord regeneration. To determine this, I have employed the use of the inverse agonists, which function by binding to receptors and produce the opposite effect of natural ligands or agonist (Kenakin, 2017). To this end, I used the inverse agonists, AM251 and AM630, which disrupt CB1 and CB2 signaling, respectively. I first used these agents to determine the functional requirement of cannabinoid signaling on gross tail and caudal spinal cord regeneration. To this end, I treated these animals in a constant treatment or a pulse treatment of the inverse agonists and recorded their growth after 7 days of regeneration. In addition, western blotting analyses were performed to observe the effect that the inverse agonists had on cannabinoid receptor expression in the regenerating tail. This was paired with indirect immunofluorescence analyses to reveal whether disrupting normal cannabinoid receptor signaling led to changes in cannabinoid receptor localization as a result of injury compared to vehicle control treated animals.

The ECS has previously been suggested to play a role in mediating bone and cartilage regeneration, at least within mammals. The natural ligands, AEA and 2-AG, have been shown to activate osteoblast formation, bone formation, and osteoclast activity (Apostu et al., 2018). Furthermore, activation of both CB1 and CB2 through the use of synthetic agonists has been shown to induce osteoblast activity and proliferation; meanwhile, treatments using inhibitors of these receptors have inhibited osteoclast differentiation (Apostu et al., 2018). In addition, CB1 is suggested to be a target during cartilage regeneration where its upregulation is essential for anti-inflammatory and chondrocyte metabolism (Lee et al., 2012). In the context of nervous system regeneration, selective activation of CB2 using the agonist, AM1241, led to dopaminergic neuron regeneration in a Parkinson's Disease model mice (Shi et al., 2017). The role of endocannabinoids as axonal guidance cues and in axonal pathfinding is also shared within regeneration-competent vertebrate species such as African clawed frogs and zebrafish; however, this has been studied in the context of early neurodevelopment, not regeneration (Zuccarini et al., 2019; Berghuis et al., 2007). Given these studies, I predict that preventing cannabinoid receptor signaling through the use of specific inverse agonists will also disrupt normal tail and spinal cord regeneration in the axolotl.

3.2. Methods

3.2.1. Animal care and surgery

All procedures and maintenance of animal is detailed in Chapter 2, Section 2.2.1.

3.2.2. Drugs and injections

AM251 (Cayman Chemical, Michigan, USA) and AM630 (Sigma Aldrich, Canada) were dissolved in a 1:1 solution of DMSO and Tween-80. Working solutions were

prepared by diluting these agents with dechlorinated water to 1μM. The animals were then bathed in these solutions constantly for a maximum of 7 days. During pulse treatments, the animals were treated in this bathing solution for 1 day then placed in dechlorinated water for the remaining 6 days of the experiment (more details in section 4.2.3). Vehicle controls for each reverse agonist were prepared using an equal concentration of 1:1 DMSO:Tween-80 (D/T80) in dechlorinated water.

3.2.3. Regeneration assay

The gross morphological analysis of regeneration extent follows the procedures as outlined in Ponomareva et al. (2015). Embryos of the Mexican axolotl were obtained through breeding. Animals used for this study ranged from 2-4 cm in length and were treated with the anesthetic, MS-222, prior to tail and spinal cord amputation using a sterile scalpel blade. Photographs were taken using a dissecting microscope equipped with Nikon DS-U2 camera and AmScope software to document the regeneration process and allow for digital measurements of caudal regeneration. The animals were maintained at room temperature (21°C) in 6 well plates bathed in 5mL of either artificial pond water, 1μM of AM251, 1μM AM630, or the vehicle control, 1:1 D/T80. For experiments requiring continual exposure of the animals in the drug treatments or vehicle control, the bathing solution was changed every other day following amputation. This differed from pulse treatments which involved exposing the animals to the drug treatment or vehicle control for 4 hours (4-hour pulse) or 24 hours (1-day pulse) immediately following amputation, then returning them to artificial pond water for the remainder of the experiment. All experiments were terminated at 7 days to avoid toxic effects of continual drug exposure. The animals were then imaged for digital measurements of length and amputated again.

The tissue collected was then fixed for immunostaining protocols or flash frozen for western blotting. At this time, animals were euthanized by prolonged exposure to MS-222. Proportional increases in body length were measured using [(Day 7 body length – amputated body length)/amputated body length] and were compared between larvae that were chemically treated and their vehicle controls.

3.2.4. Western blotting

Protocols for western blotting are detailed in Chapter 2, Section 2.2.2.

3.2.5. Immunofluorescence staining

Protocols for fixation, cryoprotection, and immunofluorescence staining are detailed in Chapter 2, Section 2.2.3.

3.2.6. Immunofluorescence Imaging

For details on immunofluorescence imaging, a detail overview is found in Chapter 2, Section 2.2.4.

3.2.7. Statistical Analyses

Data were analyzed and illustrated using Graphpad Prism 8 for Windows (La Jolla, CA, USA). Data acquired from the tail regeneration assays were analyzed using an Unpaired t-test or a One-Way ANOVA paired with a Tukey's *post-hoc* test. A One-Way ANOVA paired with a Tukey's *post-hoc* test was also used to determine statistical differences in protein expression during different stages of regeneration. A P-value less than 0.05 was used to determine statistical significance for all analyses.

3.3. Results

3.3.1. Constant treatment with inverse agonists of cannabinoid receptors significantly impaired tail and spinal growth after amputation

I observed that disrupting CB1 signaling through seven days of constant treatment in AM251 significantly decreased tail and spinal cord regeneration compared to the control treated animals ($P = 0.009$, unpaired t-test; Figure 10A). A similar decrease was observed after disrupting CB2 signaling via AM630 treatment ($P = 0.0026$, unpaired t-test; Figure 10B). The proportional increase in body length is highly correlated with tail lengthening during regeneration (Ponomareva et al., 2015) and it appeared the inverse agonist-treated animals grew to approximately 50% of the vehicle control-treated animals.

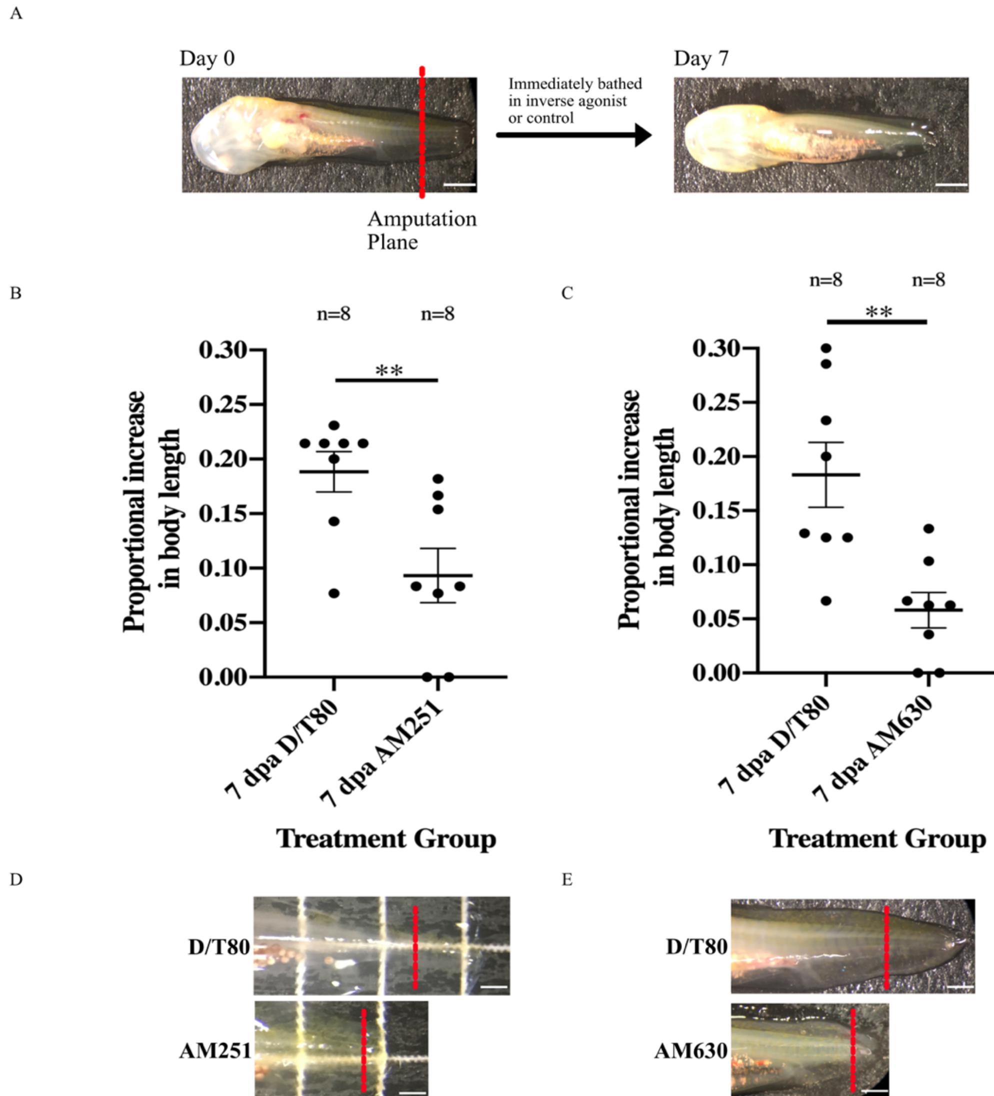


Figure 10. Constant treatment in CB1 and CB2 inverse agonists significantly inhibited caudal tail and spinal cord regeneration. A) Visual representation of the regeneration assay. Scale bar = 2mm. **B)** Proportional increase in body length following 7 day constant bathing treatment in 1μM AM251 and vehicle (1:1 DMSO:Tween-80). ** (P<0.01). **C)** Proportional increase in body length following 7 day constant bathing treatment in 1μM AM630 and 1:1 DMSO:Tween-80. ** (P<0.01). **D** and **E)** Representative images of axolotls following 7 days of reverse agonist and control treatments. Red lines indicate the original plane of amputation. Scale bar = 1mm.

3.3.2. Treatment with inverse agonists for a 1-day pulse similarly decreased tail and spinal cord regeneration at 7 dpa

My next aim was to discover if treating the axolotls with the inverse agonists for a shorter period of time was able to have a similar inhibitory effect as 7 days of constant treatment. This was to address the possibility that these inhibitory effects observed in Figure 12 could have resulted from toxic side-effects due to constant bathing treatment with the inverse agonists. The following data represent observations from animals that were treated with 4-hour and 1-day incubations in inverse agonists that were then left to develop to 7 dpa. These timepoints were selected as my previous western blotting data demonstrated a significant increase in CB1 expression as early as 4 hpa and 1 dpa, whereas CB2 was significantly increased by 7 dpa.

When the animals were treated with a 4-hour pulse of either AM251 or AM630, I did not find a statistically significant decrease in the mean proportional body length relative to the D/T80 or artificial pond water controls ($F_{(2.451, 20.42)} = 3.651$ and $P = 0.8170$; Figure 11A & 11C). However, by extending the pulse treatments to 1 day, I observed a significant inhibition in caudal tail and spinal cord regeneration ($F_{(2, 27)} = 18.86$ and $P < 0.0001$; Figure 11B & 11D). Particularly, I observed an approximate 50% reduction in mean proportional body length in animals treated with either a 1-day pulse in AM251 ($P < 0.0001$) or AM630 ($P = 0.0001$).

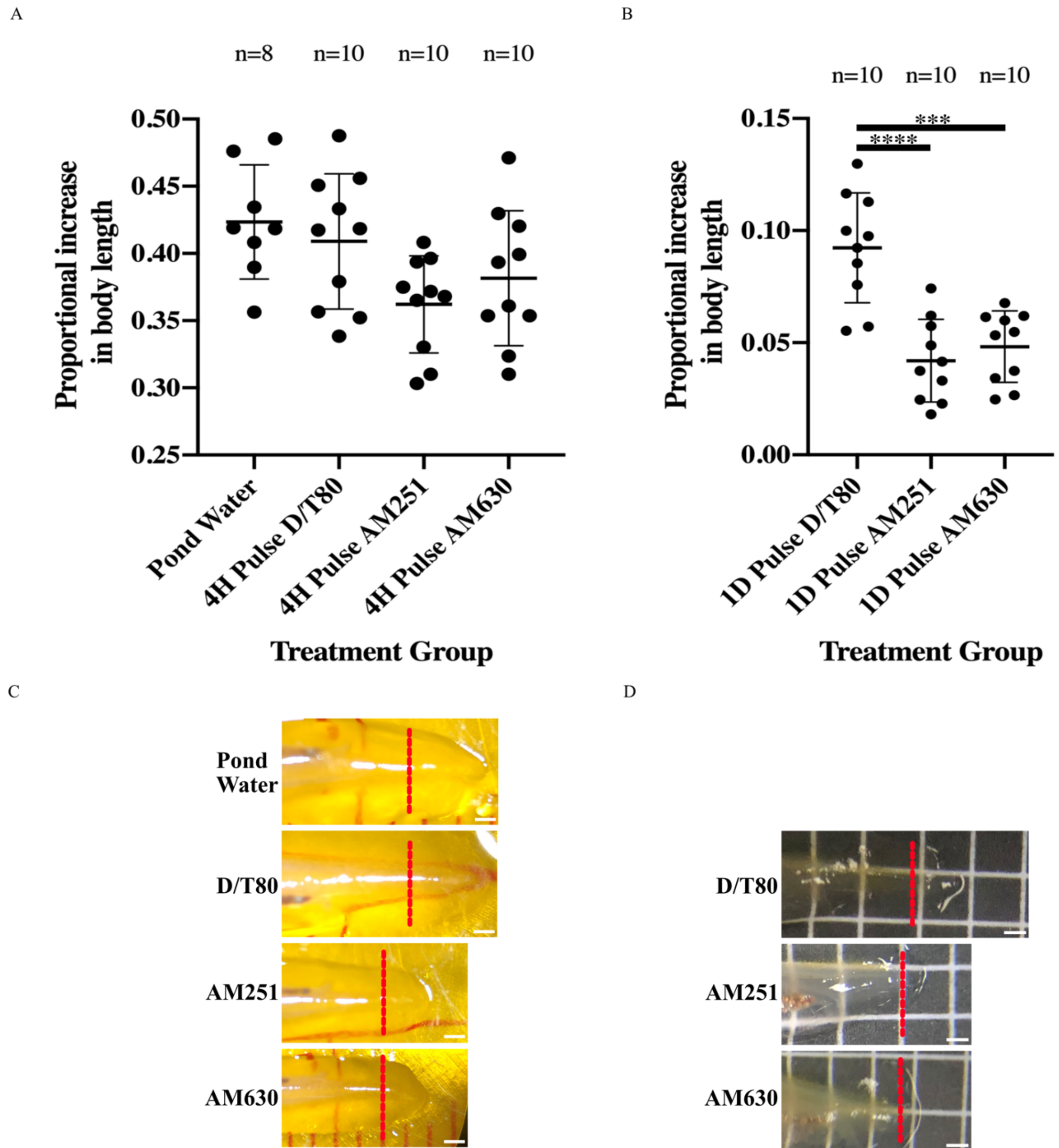


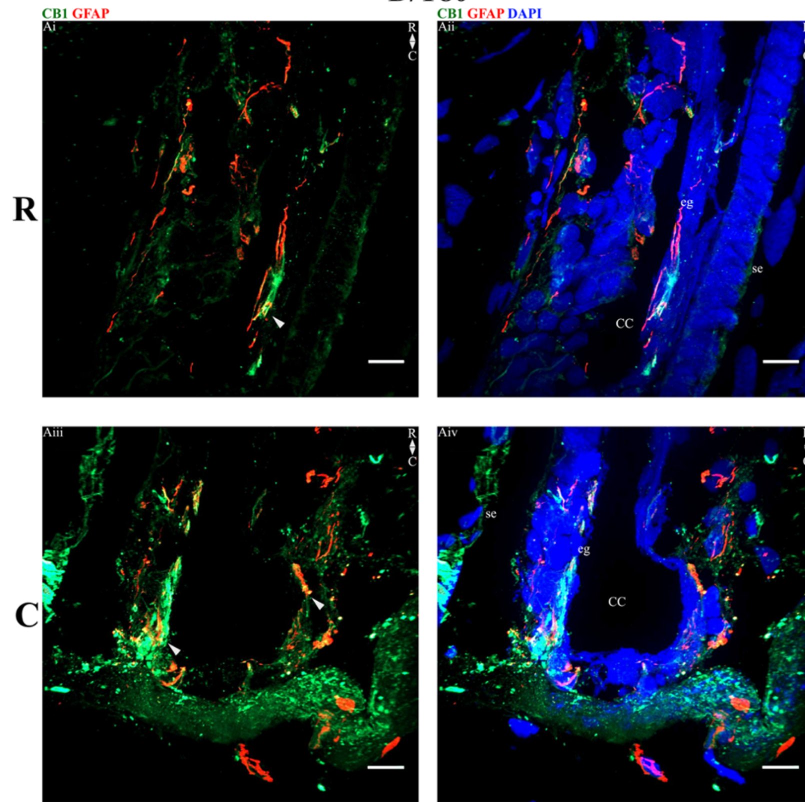
Figure 11. One Day, but not 4-hour, pulse treatments with CB1 and CB2 inverse agonists significantly inhibited tail and spinal cord regeneration. A) Proportional increase in body length following a 4-hour pulse treatment in 1μM AM251 or AM630, 1:1 DMSO:Tween-80, and pond water (dechlorinated water). **B)** Proportional increase in body length following a 1-day pulse treatment in 1μM AM251 or AM630, and vehicle control. *** ($P < 0.001$) and **** ($P < 0.0001$). **C** and **D)** Representative images of axolotls following 1 hour and 1-day pulse treatments with red line to indicate the original plane of amputation. Scale bar = 1mm.

3.3.3. Incubation with inverse agonists for 4 hours prevented upregulation of cannabinoid receptor expression in the regenerating spinal cord

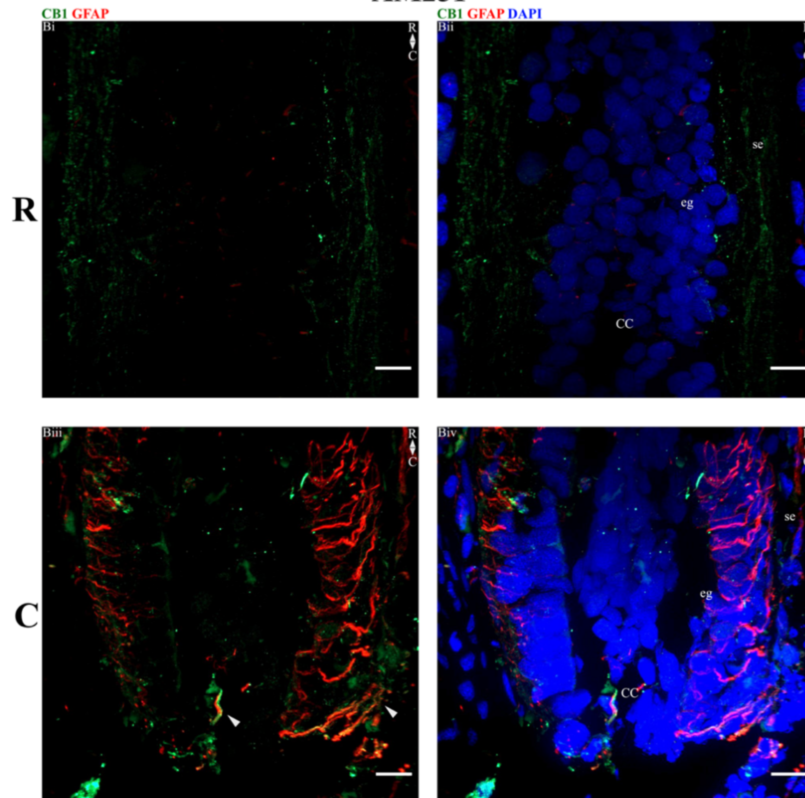
Considering that my previous western blotting data demonstrated a significant upregulation of CB1 expression beginning at 4 hpa, I next sought to investigate the effects of disrupting normal CB1 signaling for the first 4 hours after amputation on the abundance and spatial distribution of CB1.

In the caudal regions of the spinal cord from D/T80 treated animals, CB1 appears to be upregulated mainly in the central canal and the wound epithelium at 4 hpa (Figure 12iii and 12 Ciii). These animals also demonstrate a qualitative upregulation of CB1 expression in the caudal regions of the regenerating spinal cord compared to more rostral regions, as well as the UINJ tissue previously displayed in Chapter 2 (Figure 7Bii, Cii). However, through qualitative analysis it appears that 4 hours of treatment with AM251 yielded a noticeable decrease in CB1 expression. Surprisingly, this corresponded with an increase in GFAP expression in the caudal regions of the regenerating spinal cord (Figure 12 Biii and 12 Diii).

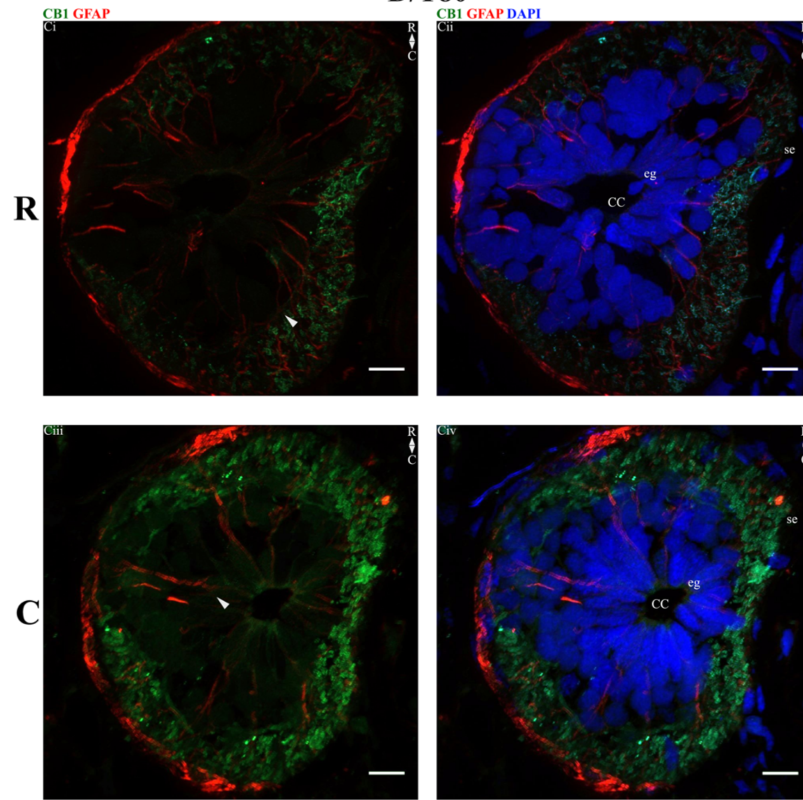
A



B



C



D

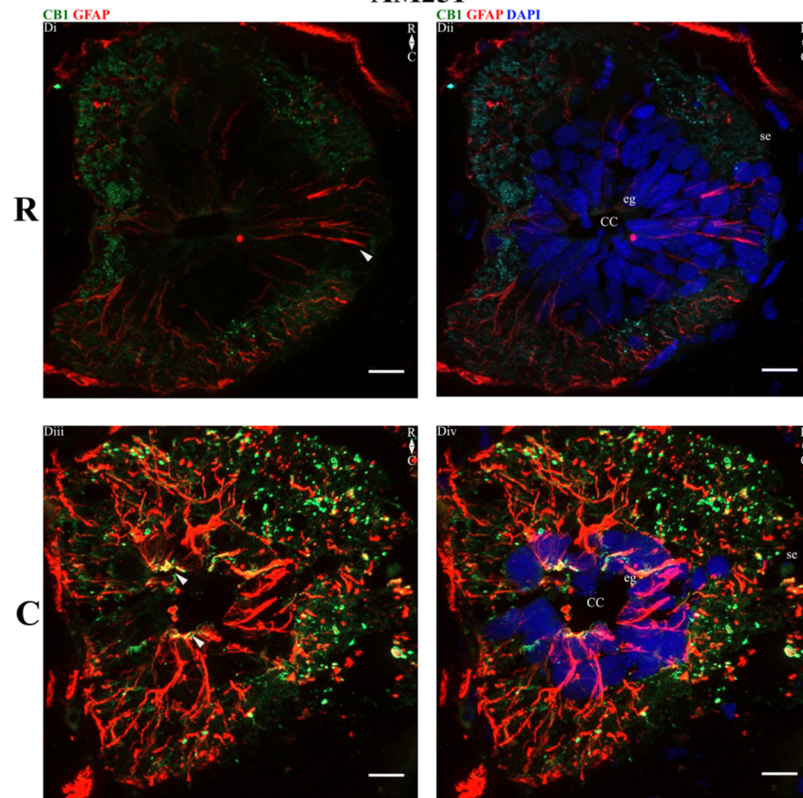
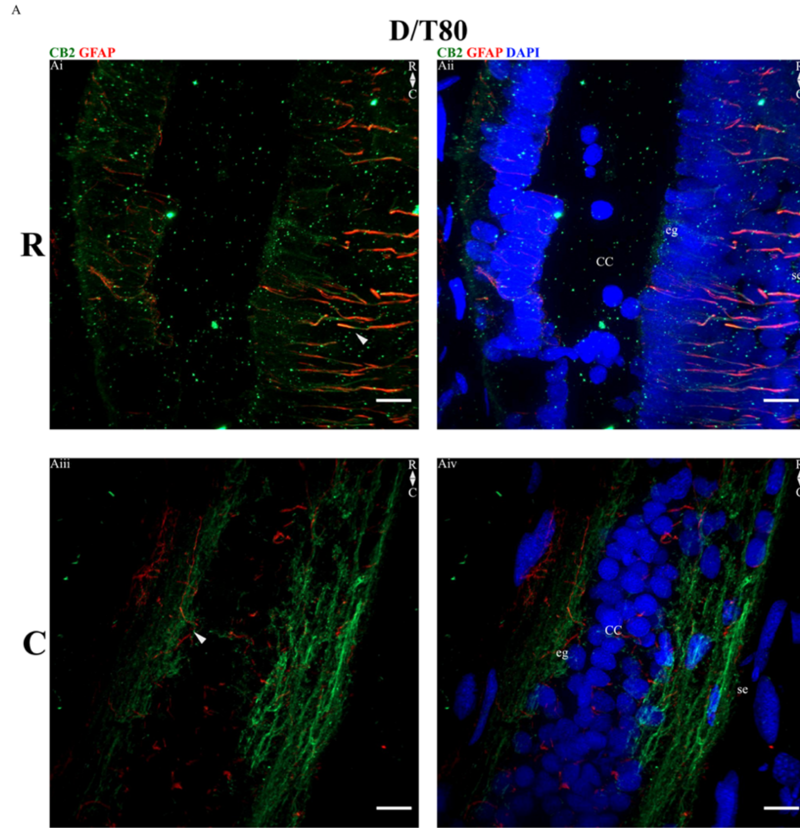


Figure 12. Application of AM251 prevents upregulation of CB1 in ependymoglial cells and wound epithelium in regenerating spinal cord at 4 hpa. A and **B)** Sagittal sections displaying the location of CB1 and GFAP protein expression in the regeneration blastema 4 hours after amputation in animals bathed in the 1:1 DMSO:Tween-80 vehicle control and 1μM AM251, respectively. **C** and **D)** Cross sections displaying the location of CB1 and GFAP protein expression in the regeneration blastema 4 hours following amputation in animals bathed in the 1:1 DMSO:Tween-80 vehicle control and 1μM AM251, respectively. The images R and C indicate whether the sections were taken rostral (R) or caudal (C) to the original amputation plane. White arrowheads indicate area of CB1 co-localization with GFAP. Scale bars for all images set at 50 μm. cc: Central canal; eg: ependymoglia; se: subependymal cells.

I next aimed to determine the effects of CB2 reverse agonist 4 on the spatial distribution of the CB2 receptor at 4 hours after amputation. The CB2 receptor was localized in the subependymal cells in D/T80 treated animals. However, qualitative analysis of these tissues did not display a large difference in total CB2 expression in the subependymal regions between the rostral and caudal sections, as well as the UINJ samples in Chapter 2 (Figure 7 Bv, Cv). There did not appear to be a downregulation of CB2 expression in AM630 treated animals compared to the vehicle control, but there was a visible upregulation of GFAP expression in caudal sections after pulse AM630 treatment (Figures 13 Biii and 13 Diii).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D/T80
CB2 GFAP
CB2 GFAP DAPI
Ai
Aii
Aiii
Aiv
R
C
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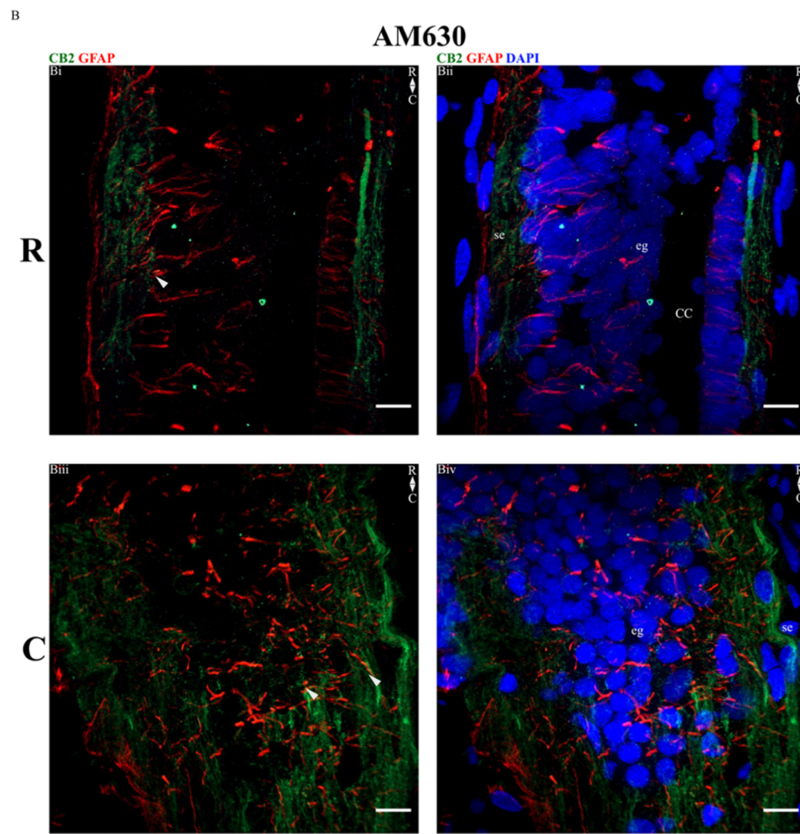
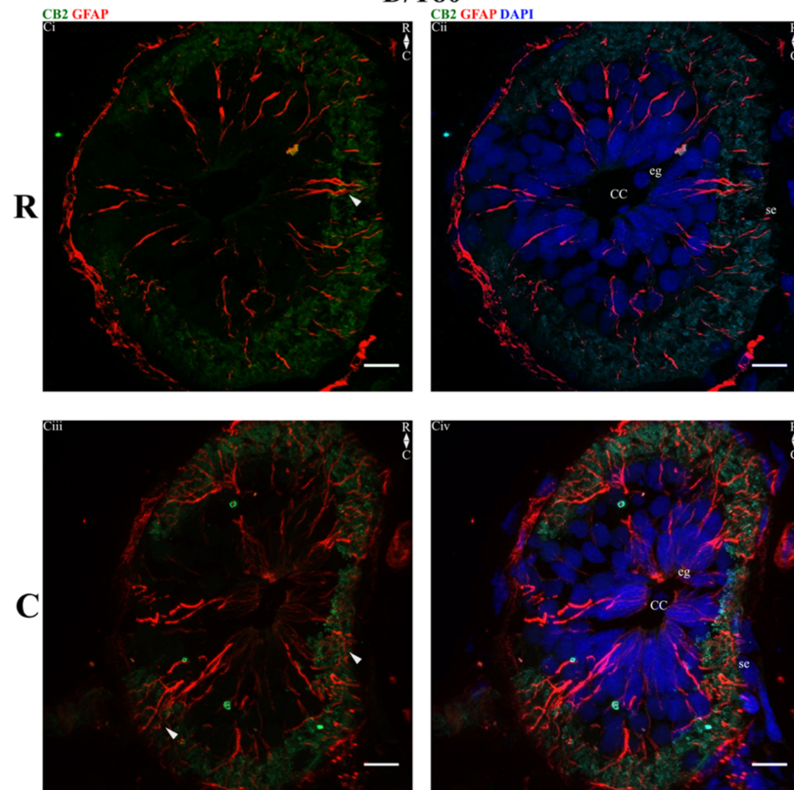
se


B
AM630
CB2 GFAP
CB2 GFAP DAPI
Bi
Bii
Biii
Biv
R
C
cc
eg
se

C



D

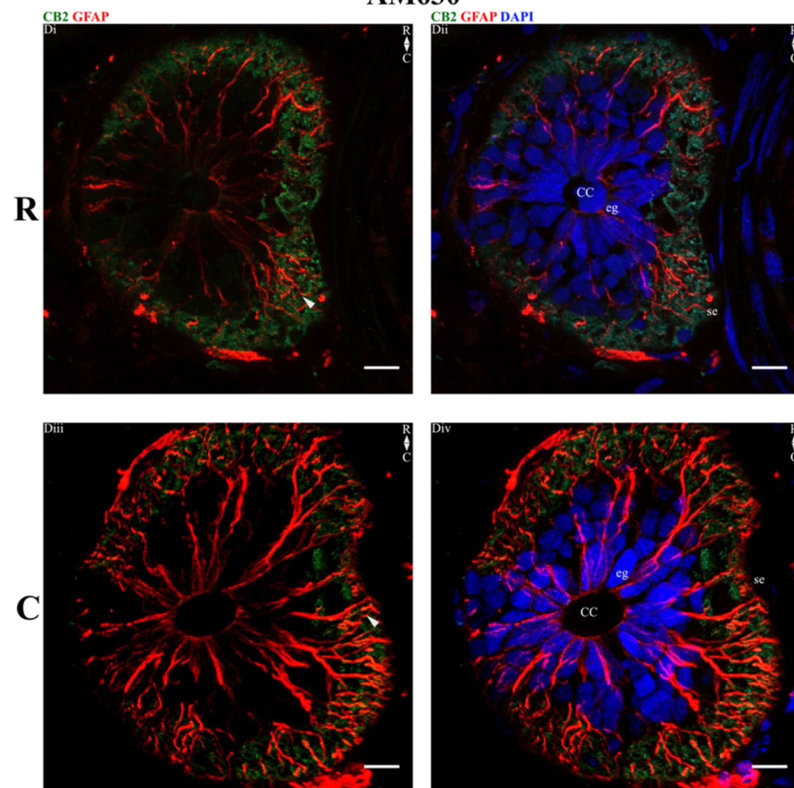


Figure 13. Application of AM630 prevents upregulation of CB2 in subependymal cells in regenerating spinal cord at 4 hpa. A and **B)** Sagittal sections displaying the location of CB2 and GFAP protein expression in the regeneration blastema 4 hours following amputation in animals bathed in the 1:1 DMSO:Tween-80 vehicle control 1μM AM630, respectively. **C** and **D)** Cross sections displaying the location of CB2 and GFAP protein expression in the regeneration blastema 4 hours following amputation in animals bathed in the 1:1 DMSO:Tween-80 vehicle control and 1μM AM630, respectively. The images R and C indicate whether the sections were taken rostral (R) or caudal (C) to the original amputation plane. White arrowheads indicate area of CB2 co-localization with GFAP. Scale bars for all images set at 50 μm. cc: Central canal; eg: ependymoglia; se: subependymal cells.

3.3.4. 1-day pulse and 7-day constant treatments with inverse agonists prevent injury-induced upregulation of cannabinoid receptor expression in the regenerating tail and spinal cord

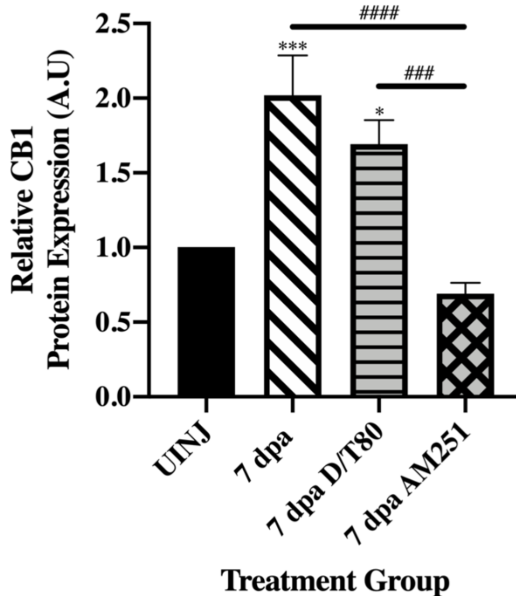
After previously demonstrating that both 1-day pulse and constant treatments in the CB1 and CB2 inverse agonists have a significant impact on normal tail and spinal cord regeneration, I aimed to identify the impact of these reagents on CB1 and CB2 protein expression using western blotting analyses. I first found that constant treatment of AM251 significantly impacted CB1 protein expression by 7 dpa ($F_{(3, 32)} = 14.69$ and $P < 0.0001$; Figure 14Ai). Specifically, CB1 expression was significantly higher in 7 dpa (no treatment) and the 7 dpa D/T80 vehicle control compared to the UINJ tissue ($P = 0.0005$ and $P = 0.0217$, respectively). Furthermore, I observed that 7 dpa and 7 dpa D/T80 resulted in significantly higher CB1 expression compared to the 7 dpa animals in constant AM251 treatment ($P < 0.0001$ and $P = 0.0006$, respectively). I found similar significant differences after performing 1-day pulse treatments using AM251 ($F_{(3, 32)} = 18.60$ and $P < 0.0001$;

Figure 14Aii). In particular, CB1 expression was significantly higher in 7 dpa and 7 dpa D/T80 when compared to UINJ tissue and 7 dpa AM251 under pulse treatments.

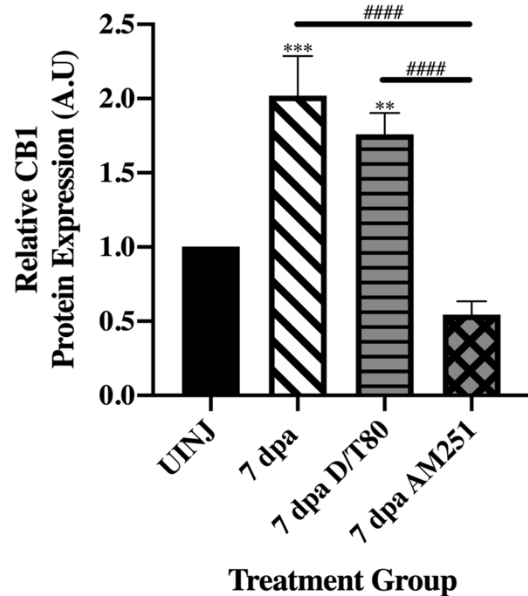
My examination into CB2 expression after constant and pulse treatments of AM630 resulted in nearly identical findings. Again, I found that CB2 protein expression was significantly impacted in both constant treatments ($F_{(3, 32)} = 24.80$ and $P < 0.0001$; Figure 14Bi) and 1-day pulse treatments ($F_{(3, 32)} = 11.60$ and $P < 0.0001$; Figure 14Bii) using AM630. Within both experiments CB2 expression in 7 dpa and 7 dpa D/T80 tissue was significantly higher compared to the UINJ and the AM630-treated tissue (Figure 14B). Thus, it appears that the effects on CB1 and CB2 protein expression from constant or pulse treatments with their respective inverse agonists are similar.

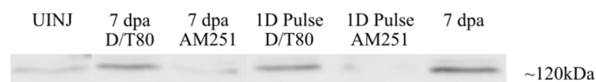
A

Ai



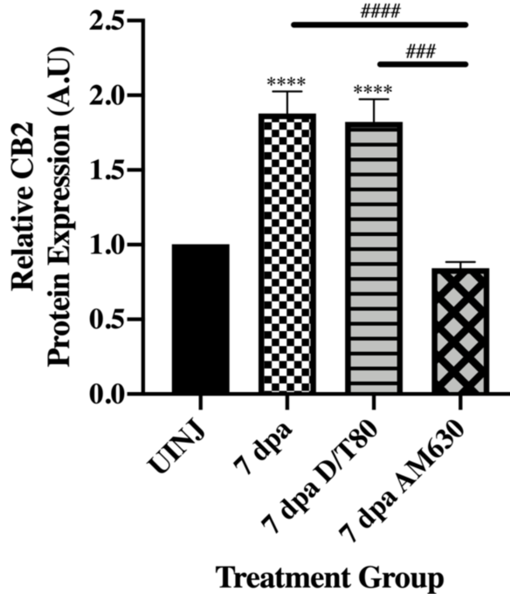
Aii



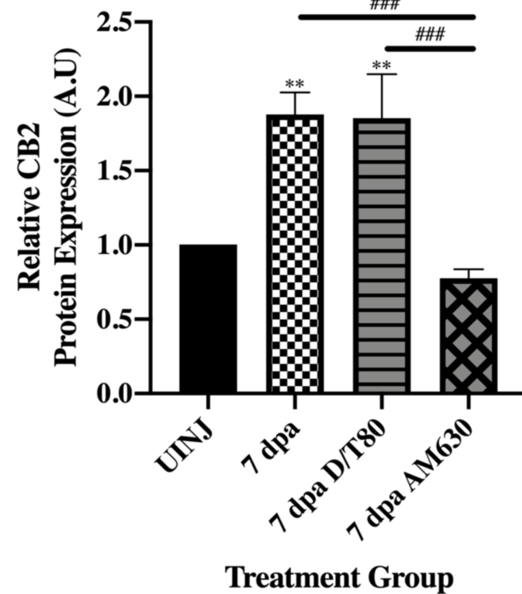


B

Bi



Bii



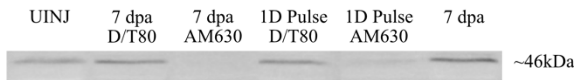
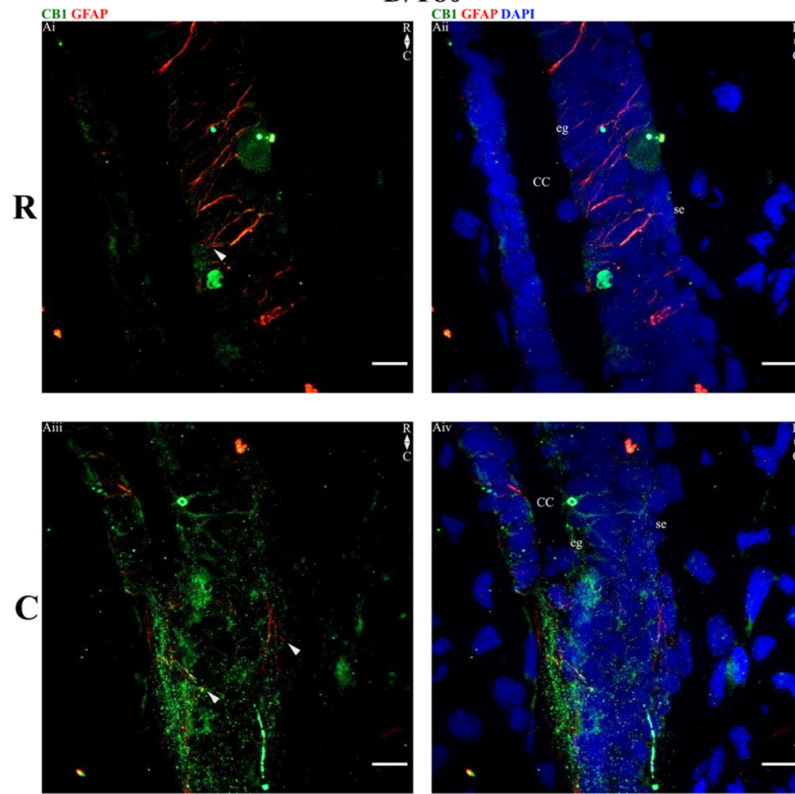


Figure 14. Constant and pulse treatments with inverse agonists prevent the normal increase in CB1 and CB2 protein expression at 7 days post tail amputation. A) Ratiometric analysis of CB1 protein expression within caudal tail regenerate tissue following tail and spinal cord amputation. Graphs Ai and Aii compare the effects of constant treatments and 1-day pulse treatments of AM251, respectively. The same 7 dpa data was used in both Ai and Aii. Treatments include 1:1 DMSO:Tween-80 and 1μM AM251, and 7 dpa tissue have no treatment. * ($P < 0.05$), ** ($P < 0.01$), *** or ### ($P < 0.001$), #### ($P < 0.0001$). **B)** Ratiometric analysis of CB2 protein expression within caudal tail regenerate tissue following tail and spinal cord amputation. Graphs Bi and Bii compare the effects of constant treatments and 1-day pulse treatments of AM630, respectively. The same 7 dpa data was used in both Bi and Bii. Treatments include 1:1 DMSO:Tween-80 and 1μM AM251, and 7 dpa tissue have no treatment. ** ($P < 0.01$), ### ($P < 0.001$), **** or #### ($P < 0.001$).

After identifying impaired caudal tail regeneration and downregulation of CB1 and CB2 receptor expression after constant treatment with the inverse agonists for 7 days, I next aimed to determine the effects, if any, of these treatments on the tissue/cellular localization of each receptor by immunofluorescence analysis. Similar to the results in Chapter 2, the vehicle controls also demonstrated an upregulation of CB1 and CB2 in the regenerate tissue caudal to the amputation plane (Figure 15Aiii & Ciii). These data also demonstrate localization of CB1 to the ependymal region, where they colocalize with GFAP+ cells, suggesting their expression within ependymoglial cells. Seven days of constant treatment in AM251, in contrast, resulted in a visual downregulation of CB1 receptor expression in the caudal regenerate (Figure 15B). Treatment with AM630 similarly prevented the normal regeneration-induced upregulation of CB2 expression in the caudal regenerate (Figure 15D).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CB1 GFAP
CB1 GFAP DAPI
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Aiv
R
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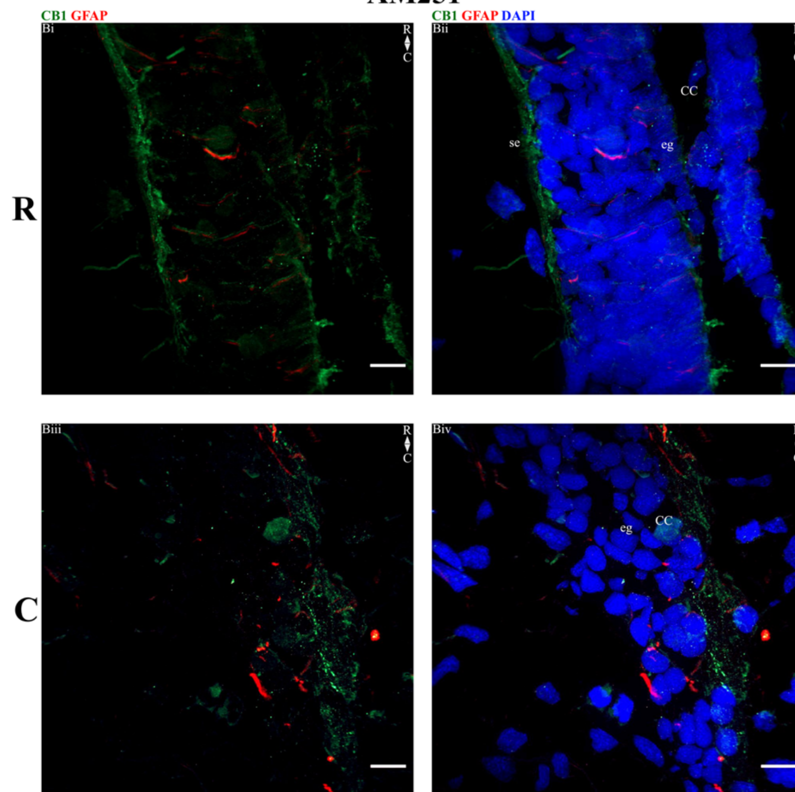
B
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CB1 GFAP
CB1 GFAP DAPI
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R
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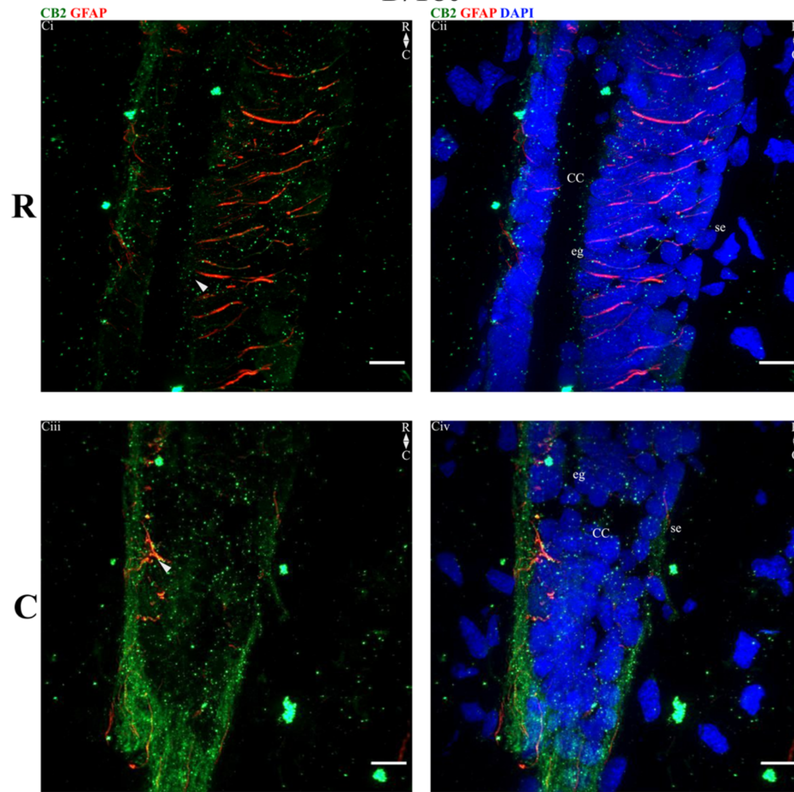
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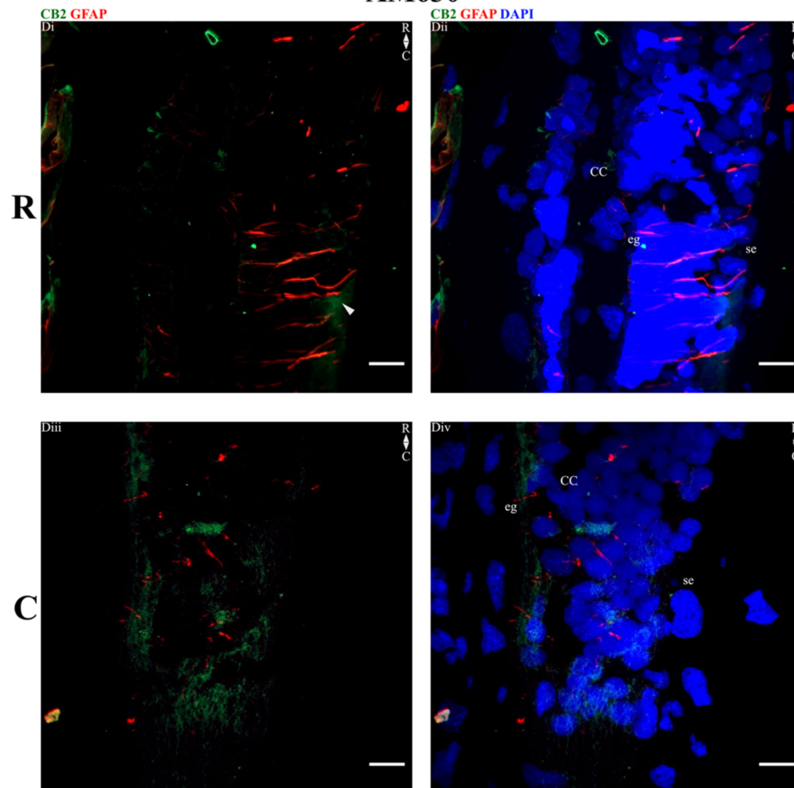
D
AM630
CB2 GFAP
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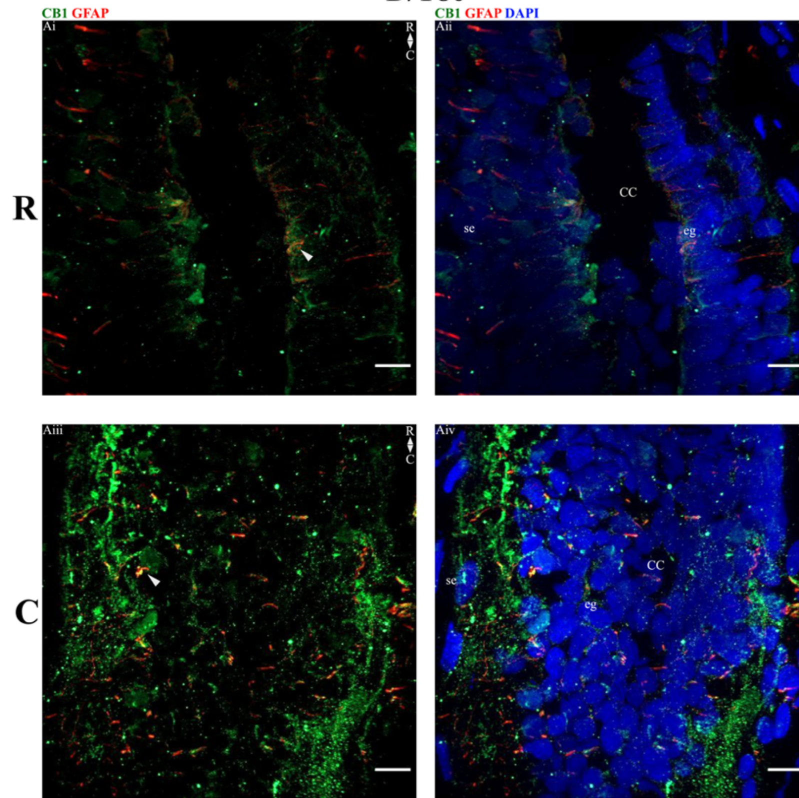
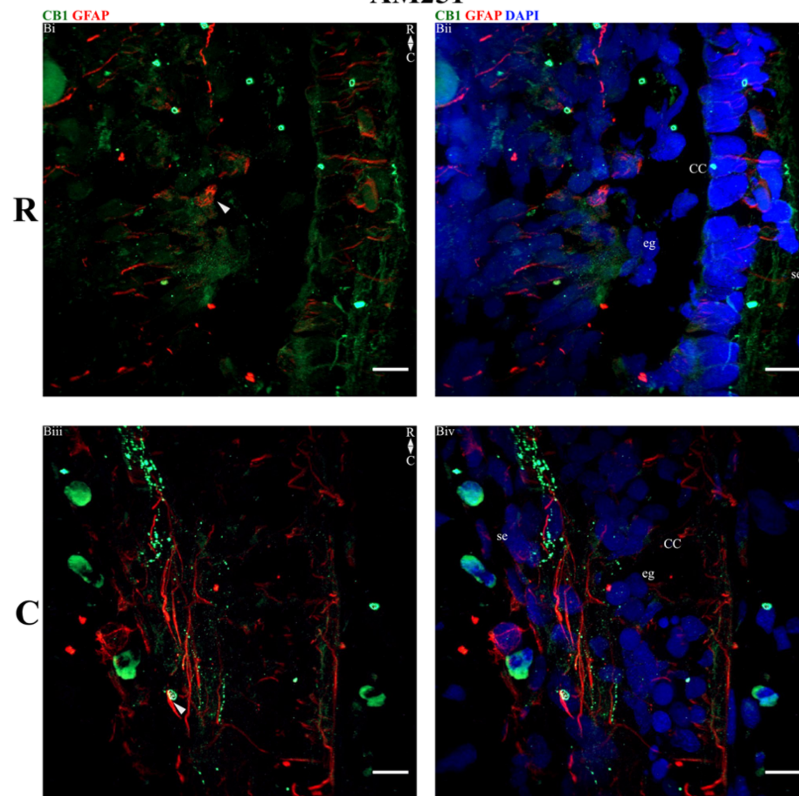
Figure 15. 7-day constant treatment with selective inverse agonists AM251 and AM630 prevented normal upregulation of their respective cannabinoid receptors, CB1 and CB2. A) Sagittal sections displaying the CB1 and GFAP localization after 7 days of constant treatment in the vehicle control, 1:1 DMSO:Tween-80. **B)** Sagittal sections displaying the location of CB1 and GFAP protein expression following 7-day constant treatment with 1μM AM251. **C)** CB2 and GFAP localization after 7-day constant treatment in 1:1 DMSO:Tween-80. **D)** CB2 and GFAP localization after 7-day constant treatment with 1μM AM630. The images R and C indicate rostral (R) or caudal (C) to the original amputation plane. White arrowheads indicate area of CB1/CB2 co-localization with GFAP. Scale bars for all images set at 50 μm. cc: Central canal; eg: ependymoglia; se: subependymal cells.

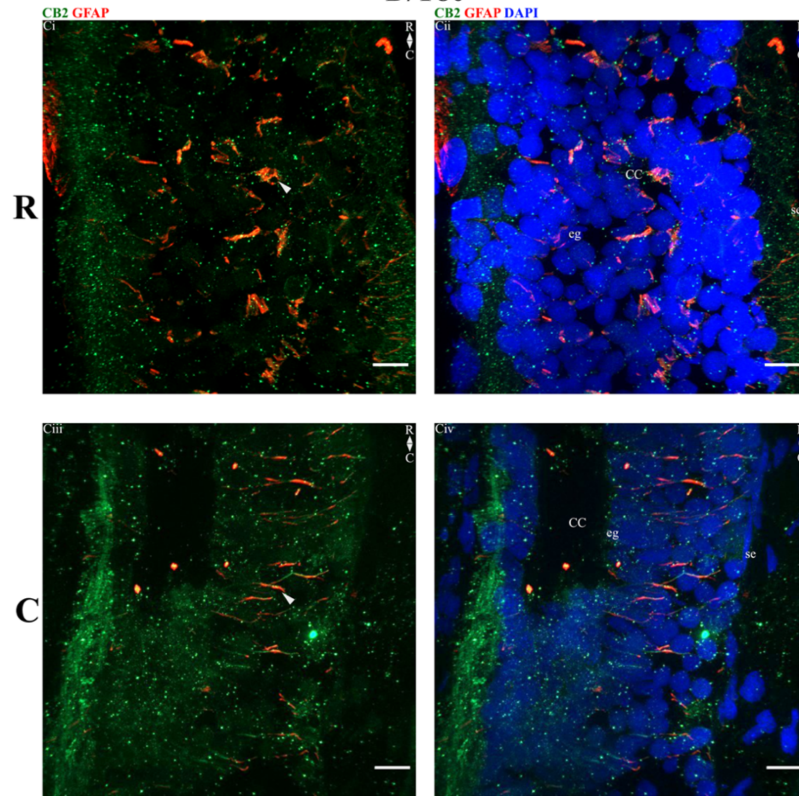
After observing the impacts on cannabinoid receptor expression in animals that were constantly bathed in the inverse agonists for 7 days, my next aim was to repeat these experiments with animals treated with the 1-day pulse protocol described previously. At 7 dpa, animals treated with the vehicle control for 1 day exhibited the normal upregulation of CB1 and CB2 expression in the caudal regenerate tissue (Figure 16A & 16C). These tissues also demonstrate the possible downregulation of GFAP expression in the tissue caudal to the amputation plane compared to tissue extracted rostral to the amputation plane. Similar to the results in the previous experiment, CB1 and CB2 expression appeared to be downregulated in the caudal tissue from animals treated with a 1-day pulse of AM251 and AM630, respectively (Figure 16B & 16D). This is most apparent in the caudal sections taken from these regenerates. In AM251-treated animals, there appeared to be an upregulation of GFAP (Figure 16Ciii). However, no such upregulation of GFAP occurred in the AM630 treated animals (Figure 16Diii).

A



B

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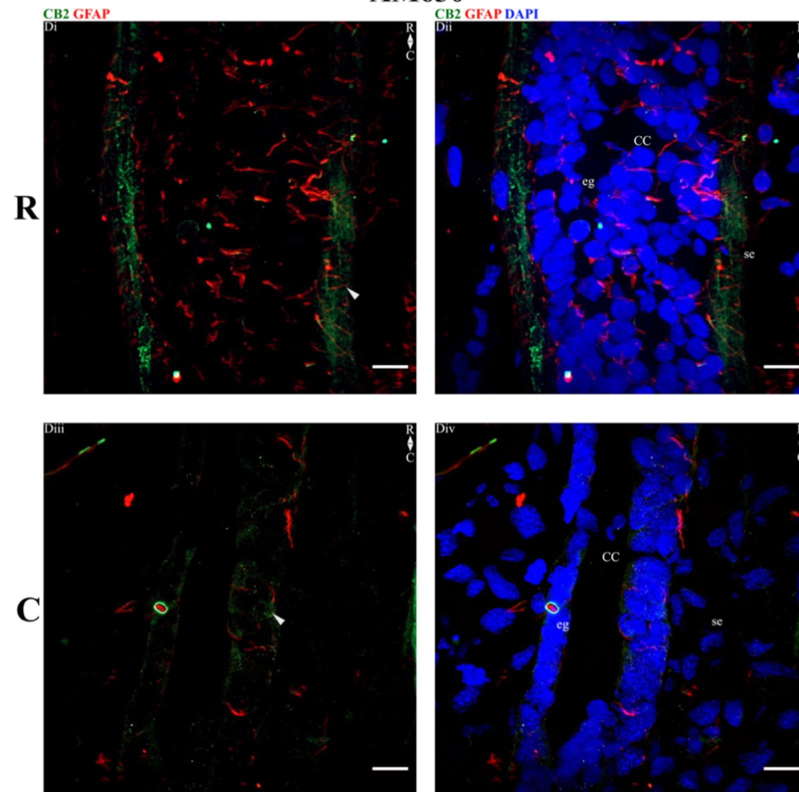
D
AM630
CB2 GFAP
CB2 GFAP DAPI
Di
Dii
Diii
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R
C
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Figure 16. 1-day pulse treatments with selective inverse agonists AM251 and AM630 prevented normal upregulation of cannabinoid receptors, CB1 and CB2. A) Sagittal sections displaying the localization of CB1 and GFAP protein expression in 7-day regenerate tissue after 1-day pulse in the vehicle control, DMSO:Tween-80. **B)** Sagittal sections displaying the location of CB1 and GFAP protein expression in 7-day regenerate tissue after 1-day pulse treatment with 1μM AM251. **C)** CB2 and GFAP localization in 7-day regenerate tissue after 1-day pulse in DMSO:Tween-80. **D)** CB2 and GFAP localization in 7-day regenerate tissue after 1-day pulse treatment with 1μM AM630. The images R and C indicate rostral (R) or caudal (C) to the original amputation plane. White arrowheads indicate CB1/CB2 co-localization with GFAP. Scale bars for all images set at 50 μm. cc: Central canal; eg: ependymoglia; se: subependymal cells.

3.4. Discussion

3.4.1. Summary of main findings

The current chapter identified a functional requirement for cannabinoid signaling through both CB1 and CB2 receptors during spinal cord and tail regeneration. I found that a 7-day constant bathing treatment in the inverse agonists of CB1 or CB2 significantly reduced caudal tail regeneration by approximately 50%. A 1-day pulse treatment with these agents had a similar inhibitory effect. No significant decrease in tail regeneration was evident after a 4-hour pulse treatment. Western blotting analysis revealed that treatment with the vehicle controls resulted in a 2-fold increase in receptor expression, similar to the non-treated 7 dpa samples. Seven-day constant treatment with the inverse agonists, however, significantly reduced the abundance of both CB1 and CB2 proteins. This was also observed after 1-day pulse treatments with the inverse agonists; however, it did not reach statistical significance for the 1-day pulse treatment with AM251 despite exhibiting nearly a 50% reduction in receptor expression. As shown previously, CB1 expression was

associated with GFAP+ cells within the ependymal region, whereas CB2 was expressed more abundantly in the subependymal region. Interestingly, results from this chapter also suggest a qualitative upregulation in the number of GFAP+ cells in animals that were treated with the inverse agonists after, amputation compared to the vehicle controls.

3.4.2. The functions of the endocannabinoid system in ependymoglial and subependymal cells

Within organisms that fail to regenerate their spinal cord, such as the rat, the receptors and the metabolic enzymes of the ECS are distributed throughout the ependymal and subependymal region, marginal glia, and the circumventricular organs (Suarez et al., 2010). It has been reported that signaling through cannabinoid receptors controls the functions of subependymal astrocytes and facilitates their roles during neurodevelopment or during neurodegenerative diseases (Fernandez-Ruiz et al., 2008; Suarez et al., 2010). For example, under conditions of neuroinflammation and ischemia, glial cell physiology and morphology are altered and the ECS will regulate the functions of microglia, astrocytes, oligodendrocytes, and ependymal cells (Massi et al., 2008).

Similar analyses of the functions of the ECS on glia in regeneration-competent organisms are scarce. However, Arafah et al. (2013) identified, in the medicinal leech, that nitric oxide (NO) release after nerve cord injury was partially under the control of endocannabinoids. NO production in the lesion site is required during nerve cord regeneration in the leech and is suggested to facilitate recruiting and stimulating microglial cells present in the lesion site (Arafah et al., 2013). However, treating the nerve cords with either AM251 or AM630 led to an inhibition of NO production and microglia recruitment in the lesion and impaired regeneration, suggesting an important role for the ECS in

regulating the recruitment of microglia expressing CB2 during regeneration. There are no current studies similar to this in urodeles or other CNS regeneration-competent vertebrates. The current literature does consist of studies performed in lamprey, frogs, and zebrafish models and provides evidence that the ECS is involved in synaptic plasticity, axonal guidance, and neurogenesis (Kyriakatos & Manira, 2007; Berghuis et al., 2007; Oudin et al., 2011; Watson et al., 2008). All of these processes are critical to regenerative success in these species.

3.4.3. The physiological relevance of GFAP+ ependymoglial cells in axolotl regeneration

The successful regeneration of the spinal cord in the axolotl, as well as other regeneration-competent vertebrates, is dependent upon the well-orchestrated processes that include the elimination of damaged cells and debris, proliferation of neural precursor cells, migration of various glia to the lesion site, neural differentiation, and reestablishment of synaptic partners (Zupanc & Zupanc, 2006; Enos et al., 2019). After the spinal cord is subjected to amputation or transection, the GFAP+ ependymoglial cells will seal the lesion and form a structure known as the ependymal bulb, the cells of which will proliferate to develop an ependymal tube to promote and guide axonal regrowth (Piatt, 1955; O'Hara et al., 1992). Under normal conditions, the number of cells that express GFAP decreases as a function of time post amputation, suggesting the loss of fibrous astrocytes (O'Hara et al., 1992). This permits the extension of axons through the lesion resulting in functional recovery and regeneration. Thus, it appears that there is a requirement to decrease the number of GFAP+ astrocytes within the lesion to permit the structural remodelling required for the establishment of the ependymal tube and subsequent axonal regrowth (O'Hara et

al., 1992). Within the current chapter, I show that normal endocannabinoid signaling is functionally required for tail and spinal cord regeneration. In addition, I also present data that showed that AM251 and AM630 significantly reduced the expression of cannabinoid receptors while appearing to greatly increase the number of GFAP+ cells, thus possibly transforming a regeneration permissive environment to an inhibitory environment. These pharmacological data suggest that, under normal conditions, the ECS may play a role regulating the ependymoglial response, ensuring the production and maintenance of a regeneration-permissive environment for neuronal outgrowth.

3.4.4. The physiological relevance of endocannabinoids on reactive gliosis

As mentioned previously, reactive gliosis is a phenomenon that is activated after damage occurs within the CNS of organisms that display limited regeneration capacity. In fact, reactive gliosis often develops into a glial scar that impedes regeneration by acting as a barrier for axonal outgrowth (Adam & Gallo, 2018). It has been consistently shown that axolotls do not produce glial scars after spinal cord injury through pre- and post-injury analyses of the main components of the glial scar including GFAP, vimentin, chondroitin sulphate proteoglycans (CSPGs), and collagens (Echeverri, 2020; Mchedlishvili et al., 2012). Thus, axons are able to regrow and re-establish functional connections. The underlying molecular pathways that result in the pro-regenerative glial cell response are not fully understood. Sabin et al. (2019), however, have recently demonstrated that upregulation of miR-200a after axolotl spinal cord injury is required to inhibit the genes that would result in the creation of the glial scar. These genes code of proteoglycans involved in cell cycle regulation and cell-matrix interactions (LGAL, DCN, BCAN), initiating inflammatory responses (TLR2), and neural growth and differentiation (CSPG5).

However, genes regulating ECM remodelling, cell migration, axon migration, and inflammation were affected as well (Sabin et al., 2019).

The participation of the ECS during reactive gliosis is not unrealistic given the role of the cannabinoid receptors in neuroprotection, inhibiting neuroinflammation, and their homeostatic roles in the CNS and the PNS via neutralization of free radical species and prevention of cytotoxicity within mammals (De Filippis et al., 2009). There is evidence from studies on human spinal cord tissue to suggest that cannabinoid signaling is involved in microglial recruitment toward dying neurons during neurodegeneration from MS or ALS (Yiangou et al., 2006). Within these microglia, CB2 was identified on the leading edge of the lamellipodia and activation of this receptor using synthetic cannabinoids resulted in suppressed inflammation in the brain and spinal cord. These data support a role for this receptor in microglial recruitment leading to decreased neuroinflammation (Yiangou et al., 2006).

There are additional pharmacological data suggesting the role of glial CB1 and CB2 receptor activation in regulating reactive gliosis. López Rodríguez et al. (2011) used a stab wound brain injury model in albino male rats and found that estradiol was able to significantly reduce the number of vimentin+ and GFAP+ astrocytes, thereby inhibiting reactive astrogliosis near the wound. However, disruption of either CB1 or CB2 activity with AM251 or AM630 inhibited the anti-glial scarring effects from estradiol administration resulting in qualitatively similar number of vimentin+ and GFAP+ cells in the lesion area, resembling the vehicle controls. Thus, estradiol may reduce reactive gliosis via activation of the ECS, at least in this specific brain injury model (López Rodríguez et al., 2011).

Adhikary et al. (2011) provide similar findings where the treatment with the CB2 agonist, O-1966, resulted in significantly improved functional recovery and a significant reduction in myeloid cell invasion and immunoreactive microglia in their murine spinal cord contusion model. Baty et al. (2008) also demonstrated that the application of a CB2 agonist, O-1966, led to partial functional recovery in a mouse spinal cord injury model. This pro-regenerative effect was attributed to decreased production of pro-inflammatory cytokines such as TNF-α as a result of CB2 activation. Thus, CB2 signaling may play a role in mediating the inflammatory responses associated with spinal cord injury. Further support for a role for the ECS in establishing a regeneration-permissive environment after injury is provided by Leichsenring et al. (2009) using a spinal nerve transection model in Wistar rats. Animals treated with either a mixture of CB1/CB2 (WIN55,212-2) agonists or a selective CB2 (GW405833) agonist showed significantly reduced astrocytic and microglial activation after surgical cord transection. However, these results were then reversed when treatment with these agonists was discontinued, suggesting that the activation of at least the CB2 cannabinoid receptor is able to prevent or reverse reactive gliosis within the transected spinal cord (Leichsenring et al., 2009).

Although these organisms fail to exhibit regeneration in the spinal cord, we might be able to extrapolate these data to organisms that do regenerate in the CNS. There are currently no studies other than my current thesis that examines the role of the ECS in spinal cord regeneration within organisms such as the axolotl. I found a qualitative increase in the number of GFAP+ cells in the spinal cord after altering normal cannabinoid receptor signaling via inverse agonist treatments. Thus, this is the first study to provide evidence

that normal cannabinoid signaling is required in regulating the pro-regenerative glial response exhibited by the axolotl.

Chapter 4. Correlating the Spatial Expression Patterns of CB1 and CB2 with Subpopulations of Proliferating and Differentiating Cells

4.1. Rationale

After observing the potential requirement for the cannabinoid receptors during regeneration, the final chapter of my thesis aimed to determine the cellular consequences of disrupting normal cannabinoid signaling. During the regeneration of the amputated tail and spinal cord, it is currently theorized that the main cellular contributors to spinal cord regeneration are the ependymoglial cells (Sabin et al., 2019; Tazaki et al., 2017; Joven & Simon, 2018). However, the factors that lead to their pro-regenerative response are not yet fully understood. Processes during development such as cell proliferation, fate determination, and neurite outgrowth, are amongst the roles that the ECS has been reported to be involved in (Aguado et al., 2006). Thus, it is possible that the ECS might be involved in these process during regeneration as well.

The purpose of this chapter was to examine whether the cannabinoid receptors were localized on proliferating and/or differentiating cell populations after tail and spinal cord amputation. It was hypothesized that the ECS plays a role in mediating proliferation of ependymoglial cells and the differentiation of immature neurons during regeneration. This is based on studies that disrupted CB1 signaling in early zebrafish development resulting in impaired cortical neurogenesis and neural progenitor proliferation (Oudin et al., 2011; Watson et al., 2008). To examine the role of the ECS in the regulation of proliferation and differentiation of ependymoglial cells in the regenerating axolotl spinal cord, a bromodeoxyuridine (BrdU) incorporation assay was performed to identify the possible colocalization of the cannabinoid receptors on proliferating cells within these tissues. In

addition, immunofluorescence analysis on the expression of doublecortin (DCX), a marker for immature neurons, was performed to assess if cannabinoid receptor expression was also found in differentiating cells.

This chapter also examines the potential downstream signal transduction pathways mediating the response of spinal cord cells to receptor activation. The majority of the current literature suggests that cannabinoid receptors mainly signal to effectors through Gi/o proteins (Dalton et al., 2009). The activation of Gi/o proteins, by either CB1 or CB2, results in the inhibition of adenylyl cyclase and the subsequent stimulation of the mitogen-activated protein kinase (MAPK) pathway (Demuth & Molleman, 2006). MAPK1/ERK2 and MAPK3/ERK1 are the two MAP kinases in the MAPK/ERK (Ras-Raf-MEK-ERK) cascade, which mediate diverse developmental functions such as cell growth, adhesion, survival, cell division, and differentiation through gene regulation (Busca, 2016). In the axolotl, sustained ERK activation is required for reentry into the cell cycle of muscle cells during limb regeneration by downregulating p53 (Yun et al., 2014). Furthermore, CB1 activation-induced MAPK signaling has previously been demonstrated to promote cell survival, neuroprotection and neurite outgrowth (Zou & Kumar, 2018). Based on these studies, I aimed to determine whether MAPK/ERK may represent a downstream effector pathway for endocannabinoid signaling during spinal cord regeneration

4.2. Methods

4.2.1. *Animal care and surgery*

All procedures and maintenance of animals are detailed in Chapter 2, Section 2.2.1.

4.2.2. Injections

Injections of bromodeoxyuridine (BrdU) were performed intraperitoneally using a sterile syringe and 30g x ½ needle 24 hours prior to scheduled amputation of tail and spinal cord tissue. BrdU (Sigma Aldrich, Canada) was dissolved in PBS to a concentration of 25 mg/ml.

4.2.3. Western blotting

General protocols for western blotting are detailed in Chapter 2, Section 2.2.2. To examine the expression of ERK and pERK over the course of regeneration, overnight incubations in primary antibodies were then performed at 4°C using 1:200 ERK1/2 (Cell signaling, Massachusetts, USA) or 1:1000 pERK (Sigma Aldrich, Canada). The following morning, the membranes were washed in 0.1%Tween-20/TBS and incubated with a secondary antibody (1:15 000) Alexa Fluor 680 goat anti-rabbit IgG (Invitrogen), or Alexa Fluor 680 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) depending on the primary antibody used, for 45 minutes at room temperature. Three biological and technical replicates were performed for each condition.

4.2.4. Immunofluorescence staining

Protocols for fixation, cryoprotection, and immunofluorescence staining are detailed in Chapter 2, Section 2.2.3. The concentrations of primary antibodies were as follows: 1:100 ACR-1 (Alomone labs, Jerusalem, Israel), 1:100 ACR-2 (Alomone labs, Jerusalem, Israel), 1:50 BrdU (DSHB, Iowa, USA), and 1:50 Doublecortin (Santa Cruz Biotechnology, Texas, USA). Negative controls were also performed by omitting the primary antibody. The following morning, the samples were washed using PBS-Triton and prepared for incubation in the secondary antibody at room temperature for 1 hour. A 1:200

dilution was used for either the Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) or the Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA). This was followed with PBS-Triton and PBS washes and application of Fluoroshield with DAPI (Sigma Aldrich, Canada) and a coverslip. Three biological and technical replicates were performed for each condition.

4.2.5. Immunofluorescence Imaging

A detailed overview of the general procedures for immunofluorescence imaging can be found in Chapter 2, Section 2.2.4.

4.2.6. Statistical analyses

Data were analyzed using Graphpad Prism 8 for Windows (La Jolla, CA, USA). A One-Way ANOVA paired with a Tukey's *post-hoc* test was also used to determine statistical significance during different stages of regeneration. P-values less than 0.05 were considered statistically significant for all analyses.

4.3. Results

4.3.1. CB1 and CB2 are expressed in both proliferating and differentiating cells of the regenerating tail and spinal cord

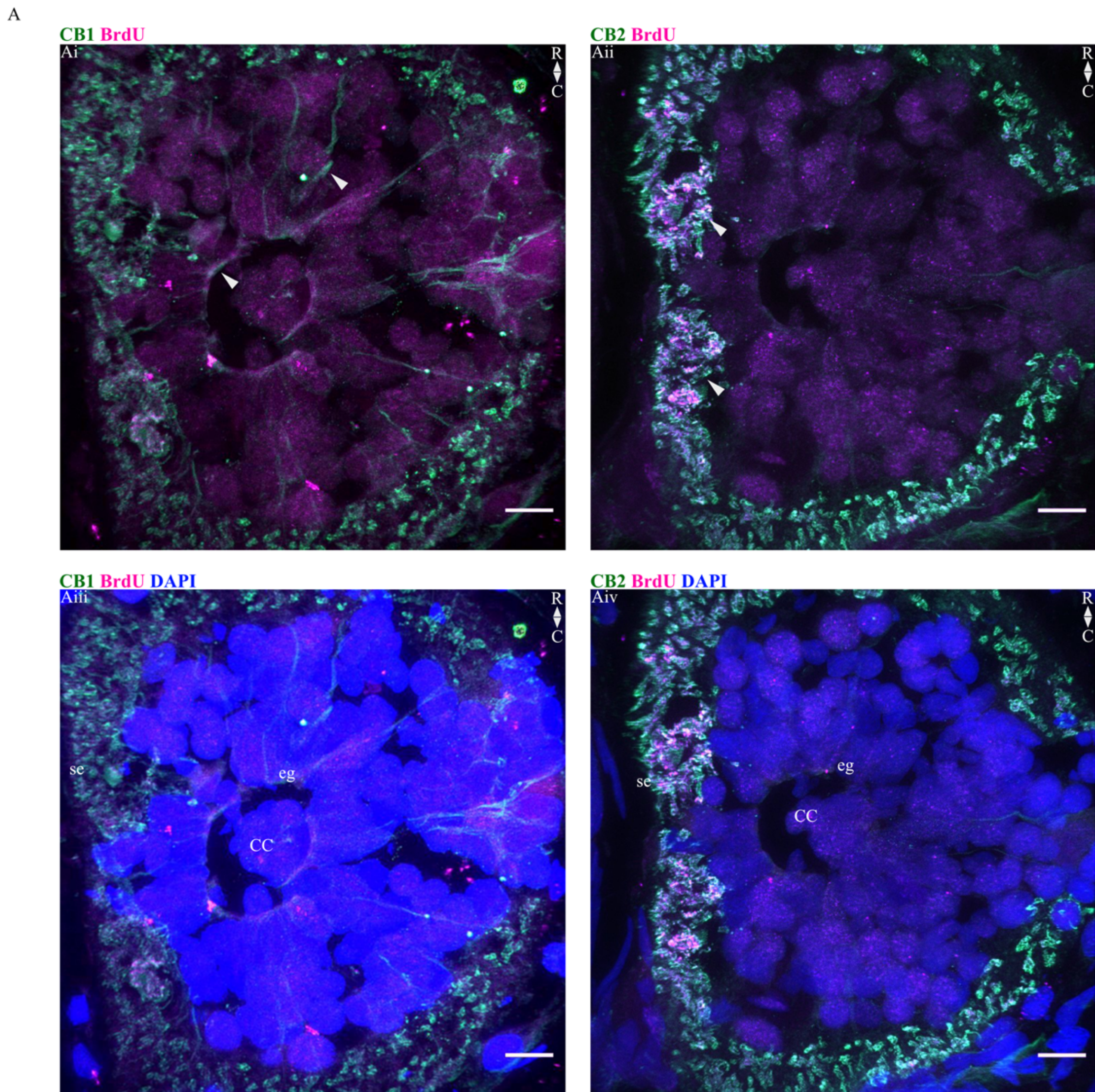
The data in the previous chapters demonstrated the upregulation of CB1 and CB2 within the regenerating spinal cord. The expression of CB1 was mainly localized within GFAP+ ependymoglial cells lining the central canal, whereas CB2 was more restricted to the subependymal regions of the spinal cord. However, this did not provide information on the proliferative or differentiative nature of these cell populations during regeneration. During regeneration, there are subpopulations of proliferating ependymoglial cells that contribute to the extension of the ependymal tube and provide cells that develop into new

neurons (Tazaki et al., 2017). Therefore, the next aim was to correlate the spatial patterns of expression of CB1 and CB2 during caudal spinal cord regeneration with subpopulations of proliferating and differentiating neurogenic cells using selective markers (BrdU and DCX, respectively) for these subpopulations.

To identify if CB1 and CB2 are expressed on proliferating cells, a BrdU incorporation assay was employed (Figure 8A). Previous data revealed that extensive proliferation occurs within the ependymoglial cells of the spinal cord as early as 4 dpa and is maintained until 8 dpa (Rost et al., 2016). My previous data also indicated a significant upregulation of both CB1 and CB2 expression during 7 dpa. For these reasons, 7 dpa regenerate tissue was used to examine the potential localization of cannabinoid receptors in proliferating cells. These data demonstrate the expression of CB1 on BrdU+ cells that line the central canal with fibrous extensions to the periphery of the spinal cord (as indicated by white arrows in Figure 17 Ai). Based on this morphology, these cells are likely ependymoglial cells (Tazaki et al., 2017). CB2 expression is also consistent with the data from the previous chapter as it resides within the subependymal region where the white areas demonstrate the overlap of CB2+ and BrdU+ cells (also indicated by white arrows in Figure 17 Aii). Thus, these data indicate that both cannabinoid receptors are expressed in proliferating cell populations in the regenerating spinal cord

This was followed by examining whether the CB1 and CB2 receptors were expressed in cells undergoing neuronal differentiation. (Figure 17B). Doublecortin (DCX) was used as it is a known marker for immature neurons and specifically marks a microtubule-associated phosphoprotein that promotes neurite extension and cell migration (Ayanlaja et al., 2017). Seven dpa regenerate tissue was also used for this experiment as

ependymal tube extension and the differentiation of new neurons from ependymoglial stem cells occurs during this period in the axolotl (Arsanto et al., 1992; Mchedlishvili et al., 2012). In 7 dpa regenerate tissue, CB1 and CB2 were also identified on DCX+ immature neurons (see white arrows, Figure 17Bi and 17Bii).



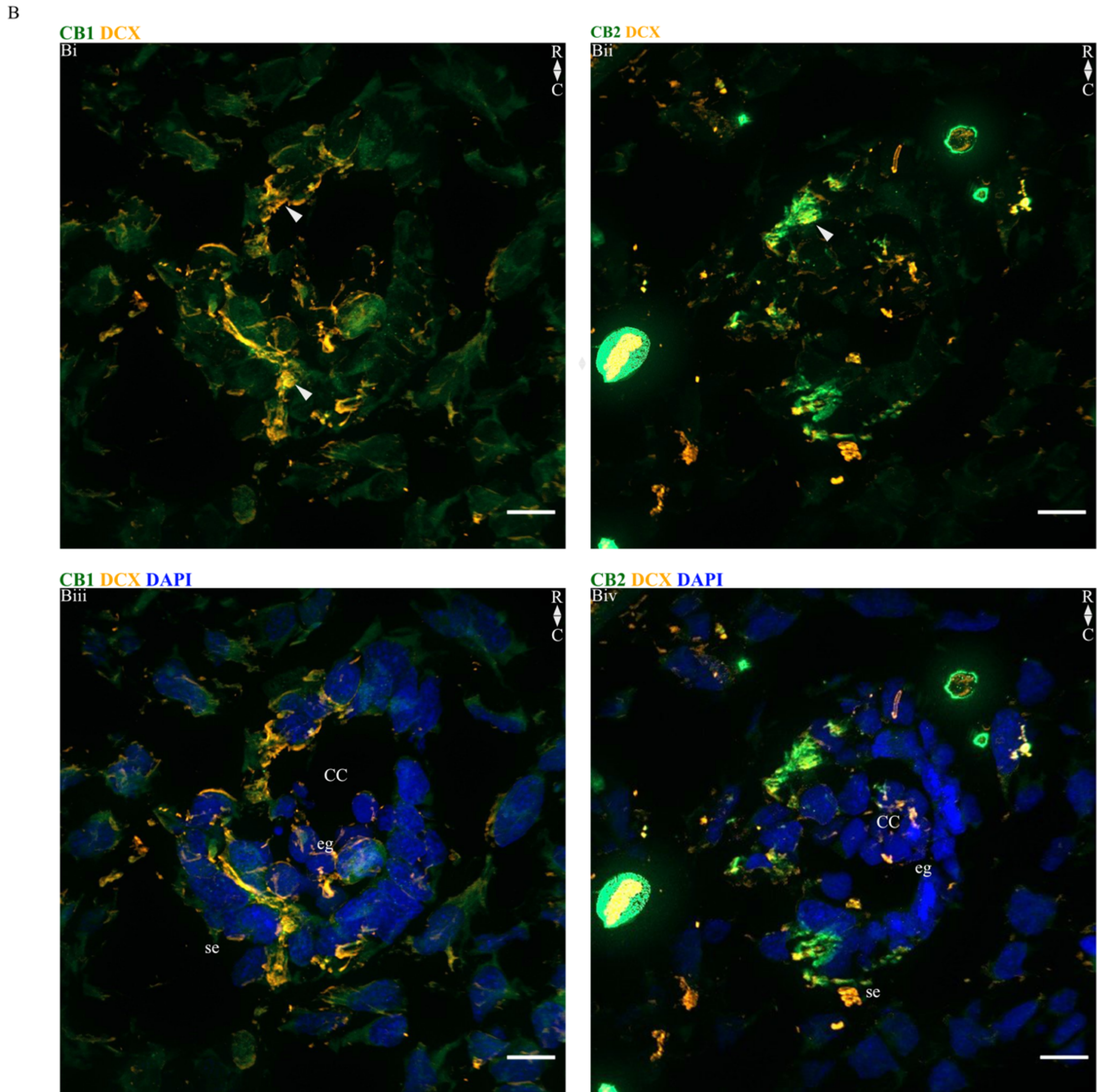


Figure 17. CB1 and CB2 are associated with dividing cells (BrdU+) and differentiating cells labelled with doublecortin (DCX+). A) Cross sections displaying the location of CB1 or CB2, and BrdU expression in 7-day regeneration blastema. **B)** Cross sections displaying the location of CB1 or CB2, and Doublecortin (DCX) protein expression in 7-day regenerate tissue. White arrowheads indicate area of CB1 or CB2 co-localization with BrdU or DCX. Scale bars for all images set at 50 µm. cc: Central canal; eg: ependymoglia; se: subependymal cells. N = 3 for biological and technical replicates.

4.3.2. Early downregulation of pERK1/2 is associated with tail and spinal cord regeneration

We next aimed to determine whether the downstream target of endocannabinoid signaling, the MAPK pathway, is altered following tail and spinal cord amputation. Between 4 hpa and 3 dpa, the one-way ANOVA identified that there were no changes in total ERK expression ($F_{(4, 30)}$ = 1.620 and P = 0.1950) (Figure 18A). This differed from pERK expression which was found to be significantly downregulated in the first 3 dpa compared to UINJ tissue ($F_{(4, 40)}$ = 6.729 and P = 0.0003). There was a statistically significant decrease in pERK expression at 1 dpa (P = 0.0237), 2 dpa (P = 0.0004), and 3 dpa (P = 0.0085), as well as between 4 hpa and 2 dpa (P =0.0193) (Figure 18B). When examining 7 dpa and 14 dpa, all the changes in protein expression seemed to diminish and no significant changes were observed in either total ERK expression ($F_{(2, 24)}$ = 3.075 and P = 0.0647) (Figure 18C) or pERK expression ($F_{(2, 24)}$ = 2.84 and P = 0.0781) (Figure 18D).

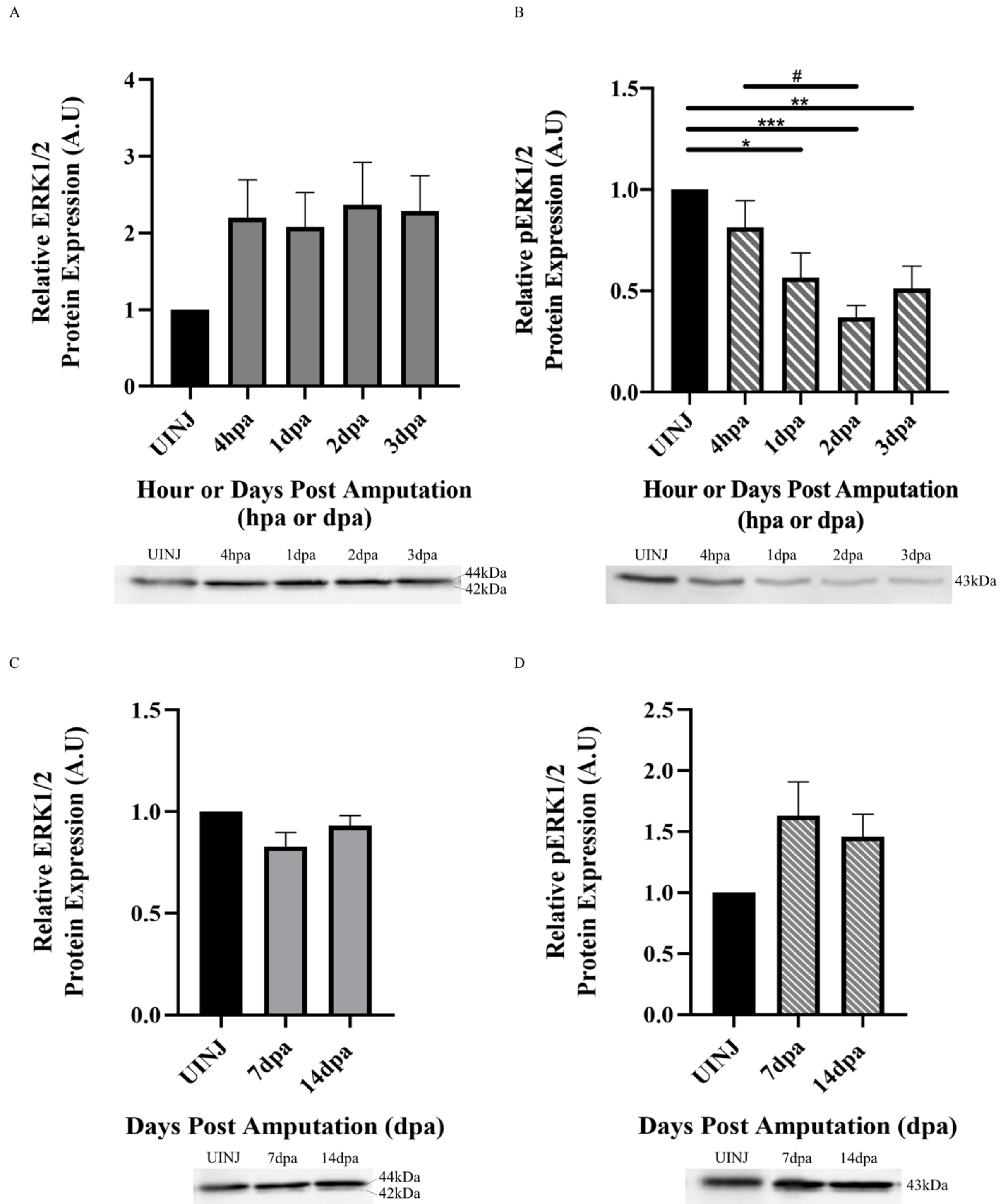


Figure 18. pERK1/2 protein expression significantly decreases shortly after tail and spinal cord amputation. A) Ratiometric analysis of total ERK1/2 protein expression within caudal tail regenerate tissue during early stages of regeneration. **B)** Ratiometric analysis of pERK1/2 protein expression within caudal tail regenerate tissue during early

stages of regeneration. **C)** Ratiometric analysis of total ERK1/2 protein expression within caudal tail regenerate tissue during later stages of regeneration. **D)** Ratiometric analysis of total pERK1/2 protein expression within caudal tail regenerate tissue during later stages of regeneration. * or # ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$). N=3 biological and technical replicates for each condition.

4.4. Discussion

4.4.1. Summary of main findings

Rost et al. (2016) and Rodrigo-Albors et al. (2015) identified a highly proliferative ependymoglial zone in the regenerating axolotl spinal cord beginning at 4 dpa and lasting until at least 8 dpa. Based on this information, I examined the potential colocalization of the cannabinoid receptors with markers indicative of proliferation (BrdU+) or immature neurons undergoing differentiation (DCX+) at 7 dpa. I found that CB1 and CB2 upregulation was exhibited on BrdU+ and DCX+ cells, suggesting the expression of these receptors on proliferating and differentiating subpopulations during regeneration. I also examined the expression of ERK and pERK as possible downstream effectors of endocannabinoid signaling during the first two weeks following tail and caudal spinal cord amputation. I identified a significant decrease in pERK expression but no statistically significant change in total ERK levels during the first 3 dpa. By 7 and 14 dpa, these differences are diminished and not significantly different from those in uninjured tissue (0 dpa).

4.4.2. Physiological relevance of cannabinoid receptor expression in proliferating cells in the nervous system

The current study focused on identifying cannabinoid expression on proliferating and differentiating cells. These data alone are correlative, and do not suggest that

cannabinoid signaling is required for proliferation and differentiation. Genetic manipulations or pharmacological experiments that would either ablate or enhance the activity the ECS were not employed in these experiments. However, there is ample support in the literature for the ECS regulating proliferation during embryonic nervous system development and within adult neurogenic niches.

The activity of the ECS during early neural development in mammals is well cited (Díaz-Alonso et al., 2012). There is evidence showing the expression of both cannabinoid receptors on embryonic stem cells (Jiang et al., 2007) and the induction of NSC and precursor cell proliferation *in vitro* through agonist-induced cannabinoid receptor activation (Molina-Holgado et al., 2007; Trazzi et al., 2010). The vital role of the ECS for regulating proliferation was further supported by Aguado et al. (2005) where they demonstrated CB1 KO mice were unable promote cell proliferation and generation of *in vitro* clusters of NSCs known as neurospheres, but an increase in proliferation in cells deficient in the degradative enzyme, FAAH. This is similar to evidence obtained from a CB2 KO mouse model wherein embryonic cortical neural progenitor cells (NPCs) also exhibited a reduced self-renewal rate (Palazuelos et al., 2011). Similar findings have also been demonstrated in pyramidal cell progenitors during embryonic neurogenesis within the ventricular/subventricular zones in the rat (Mulder et al., 2008).

The active role of the ECS during proliferation is also evident postnatally within neurogenic niches. The neurogenic niche is a microenvironment that supports NSCs and their progeny (Rodrigues et al., 2019). This microenvironment provides these cells with various signals that influence their behaviour to proliferate or differentiate. Adult NSCs reside in these niches and it is well known that these adult NSCs utilize a functional

endocannabinoid system (Zimmermann et al., 2018). In fact, CB1 expression is evident in all adult neurogenic regions of rodents (Paniagua-Torija et al., 2015). Similar to embryonic development, the ECS has recently been shown to regulate NSC proliferation in the subventricular zone and subgranular zone of the adult brain as well (Rodrigues et al., 2019). This correlates with several studies that suggest the modulatory role of cannabinoids on the NSCs in the adult mammalian brain (Prenderville et al., 2015). The activation of CB1 using the CB1 agonist, R-m-AEA, also induced proliferation and cell renewal in the mouse subventricular cell cultures (Xapelli et al., 2013). These results, however, were reversed when incubated with the CB1 inverse-agonist, AM251 (Xapelli et al., 2013). Genetic knockout mice by Zimmermann et al. (2018) further support the requirement of CB1 for proliferation. In their model, Zimmermann et al. (2018) inactivated CB1 in NSCs, which inhibited their proliferation and reduced the number of newborn neurons, thus supporting a role for ECS signaling through CB1 during neurogenesis in the adult rat. In the spinal cord of rodents, there are NSC populations present in the ependymal region. As mentioned in previous chapters, this region surrounds the central canal and these cells express significant levels of CB1 ($CB1^{HIGH}$ cells) and are induced to proliferate upon receptor activation (Paniagua-Torija et al., 2015). Furthermore, it has been suggested that CB1 signaling was associated with regulating the number of cells undergoing proliferation during neurogenesis, especially after brain insults (Rivera et al., 2011).

CB2 activation is also implicated in the regulation of proliferation of cells within neurogenic niches. The role of CB2 in regulating proliferation is supported through studies using the agonist, HU-308, which induced the proliferation of NSCs derived from embryonic cortices and adult subventricular zones of mice through PI3K/Akt/mTOR

dependant signaling *in vitro* and *in vivo* (Palazuelos et al., 2006). There are also studies in which genetic alteration of the enzymes involved in synthesizing or degrading endocannabinoids altered the rate of NSC proliferation (Goncalves et al., 2008). Goncalves et al. (2008) further suggested the requirement for CB1 and CB2 in NSCs as they demonstrated that inhibiting these receptors using either AM251 (a CB1-specific inverse agonist) or AM630 (a CB2-specific inverse agonist) significantly reduced proliferation of these cells *in vitro*.

Data are far less available in terms of the involvement of the ECS in cell proliferation after injury and within regeneration-competent animals but, Lam et al. (2006) demonstrate the presence of CB1 in the telencephalic periventricular matrix of zebrafish leading them to suggest its role in neurogenesis. However, not much else has been identified in terms of moderating proliferative events in regeneration-competent organisms.

4.4.3. Physiological relevance of cannabinoid receptor expression in differentiating cells in the nervous system

The current study has shown CB1 expression in (DCX+) immature neurons. Xapelli et al., (2013) similarly demonstrated the detection of CB1 in Nestin+ immature neurons and astrocytes derived from mouse NSCs. Furthermore, CB1 activation through AEA induced differentiation of NSCs into corticospinal neurons and the initial development of dendrites on these neurons (Tapia et al., 2017; Rodrigues et al., 2019). This was further supported by Compagnucci et al. (2013) where they demonstrated that CB1 activation using ACEA, induced differentiation of neural precursor cells towards a neuronal lineage in mice.

The involvement of cannabinoids also extends to astrogenesis and oligodendrogenesis in the early postnatal brain where CB1 activation promotes astrocyte differentiation in mouse neural progenitor cells *in vitro* and *in vivo* (Aguado et al., 2006). Differentiation into oligodendrocytes has been cited in the literature through non-selective activation of the cannabinoid receptors using WIN 55,212-2, an agonist that activates both CB1 and CB2 (Tomas-Roig et al., 2016), and through selective activation of either CB1 or CB2 (Gomez et al., 2010). From these data, we can extrapolate that CB1 may be expressed to regulate differentiation during regeneration as well.

The literature seems to suggest that CB1 may play a larger role than CB2 during neural differentiation. In the developing nervous system, functional CB2 receptors are identified on NSCs and NPCs (Aguado et al., 2005; Rodrigues et al., 2019). However, Palazuelos et al. (2011) observed a significant reduction of CB2 expression *in vitro* while culturing HiB5 cells in differentiating conditions. This is further supported through RT-PCR and western blotting analyses performed by Compagnucci et al. (2012) which indicated that CB1 is more abundantly expressed than CB2 in differentiating cells. This was shown to have physiological relevance as these researchers observed that the natural ligand, AEA, and the CB1-specific agonist, ACEA, both induced differentiation of NSCs into neurons. However, this was not found with the CB2-specific agonist, JWH-133, as it did not induce differentiation. Furthermore, administration of ACEA also promotes the maturation of neurons during differentiation (Compagnucci et al., 2012). Together, these data suggest that although CB2 expression is required during the proliferation of NSCs, it is potentially less important for cells undergoing differentiation.

4.4.4. Potential downstream effectors of cannabinoid signaling

After acquiring correlative data suggesting the expression of CB1 and CB2 on the proliferating and differentiating cells in the regenerating axolotl spinal cord, I next aimed to discover what signal transduction pathways downstream of cannabinoid ligand/receptor binding and activation of the respective G-proteins may be responsible for these potential processes. Cannabinoid receptor activation, particularly CB1, is mainly reported to inhibit adenylyl cyclase, leading to decreased cAMP, thereby leading to the activation of the MEK/ERK and PI3K/AKT pathways (Lu et al., 2015). Notably, the MAPK pathway has been previously implicated in regulating cell proliferation, differentiation, and apoptosis during development (Dalton et al., 2009). Furthermore, there is evidence from Yun et al. (2014) to suggest that sustained ERK activation is required for reentry into the cell cycle of muscle cells during axolotl limb regeneration. Thus, I focused on the determining the injury-induced impacts on ERK and pERK in the regenerating axolotl tail and spinal cord.

Various studies have identified the activation of ERK in neurons, microglia, and astrocytes after a wide variety of injury models in the nervous system (Ma & Quirion, 2005). Furthermore, Sabin et al. (2015) provide evidence of the injury-induced ERK activation (increased pERK expression) in the ependymoglial cells of the regenerating axolotl spinal cord, leading them to suggest that pERK activates cFOS to direct the ependymoglial cells towards a regenerative response. Thus, it was initially expected that pERK expression would be observed following tail and spinal cord amputation. Surprisingly, I observed a decrease in activated pERK expression during the first three days post tail amputation. There was no statistically significant change in total ERK

expression during either this period, or at 7 and 14 dpa. Moreover, the downregulation of pERK at 3 dpa was not evident at 7 and 14 dpa.

Despite these findings, there are possible reasons and considerations for the observed decrease in pERK. ERK activation may be involved in regulating neuropathic pain according to mammalian studies where ERK inhibition using the kinase inhibitor, U0126, inhibited spinal nerve ligation-induced mechanical allodynia (Obata et al., 2004). Song et al. (2005) also suggested that the function of pERK after injury in the spinal cord may partially be due to cAMP response element binding protein (CREB)-dependent gene expression. Using a rat chronic constriction injury (CCI) model, either U0126 treatment or ERK antisense ODN inhibited the upregulation of pERK, pCREB, and c-Fos expression in the spinal cord they previously injured through CCI. Song et al. (2005) concluded that ERK activation contributed to allodynia and hyperalgesia. Thus, the downregulation of the ERK pathway may be a method of neuropathic pain attenuation in the axolotl, and it is possible that this could be related to the suggested anti-allodynic and anti-hyperalgesic effects of CB1 expression previously suggested in Chapter 2.

There are also studies that suggest ERK1/2 signaling may aid in recovery from spinal cord injury in mammals. ERK signaling has been implicated in neurite outgrowth where miR-133b activated MEK/ERK and PI3K/AKT pathways via suppression of RhoA in rat cell cultures (Lu et al., 2015). In addition, Zhang et al. (2014) found that SCI recovery in rats induced from NGF administration was due to decreased neuronal apoptosis via ERK1/2 and Akt/GSK-3β activation. Lu et al. (2007) demonstrates, using a rat SCI model, that the inhibition of ERK phosphorylation via the application of the kinase inhibitor, U0126, resulted in greater functional recovery than the vehicle control 3 hours after injury.

Lu et al. (2007) observed lower levels of microglial infiltration and tissue damage in the treatment group, resulting in greater neuronal cell survival at 3 and 7 days after injury when compared to the non-treatment group. These authors also suggest that ERK1/2 phosphorylation occurs within the first few hours after tissue damage based on previous experiments. Furthermore, it is possible that microglial infiltration is modulated by CB1 during this period of spinal cord regeneration, as CB1 is also known to be expressed on microglia, at least in mammals (Stella, 2010). Thus, it is possible that the significant decrease in pERK1/2 expression observed in the current study might regulate microglial and immune cell infiltration after tail and spinal cord amputation.

In addition, it is possible that ERK activation might be associated with reactive astrocytes after injury in the spinal cord. Carbonell & Mandell (2003) demonstrate using a mouse forebrain stab lesion model the activation of ERK in GFAP+ astrocytes more proximal to the lesion, suggesting functional activation of these cells. CREB was also identified in perilesional glia, further suggesting a role for this cascade in astrogliosis. Rapid activation and spreading of astrocyte ERK have been identified during human reactive gliosis using their *in vitro* model (Mandell et al., 2001). Although these authors did not fully conclude that the reactive gliosis phenotypes are under the control of ERK activation, they do suggest the possibility that ERK activation may induce GFAP expression as the GFAP 5'-promoter contains the consensus AP1 response element where pERK may bind and enhance GFAP expression after injury. Thus, it is possible that the downregulation in pERK I observed in my study may be involved in the pro-regenerative response of glia in the axolotl.

Furthermore, the decrease in pERK expression might be associated with the upregulation in CB1 observed from 4 hpa – 3dpa. It is possible that changes in cannabinoid signaling in response to injury may alter ERK activation. The MAPK signaling pathway has been well-documented for its association with CB1-activation in terms of regulating the cell cycle, cell proliferation, and cell death (Zou & Kumar, 2018). Latini et al. (2014) also demonstrate that CB2 activation with the agonist, JWH-015, improved functional spinal cord recovery and delayed neural degeneration through ERK1/2 inactivation in a rat spinal cord hemisection model. Further investigation identifying potential alterations in the ERK and pERK expression patterns within regenerating animals treated with an inverse agonist for CB1 of CB2 is necessary to decipher whether this downstream pathway is, in fact, mediated by the ECS during spinal cord regeneration.

Chapter 5. General Discussion and Conclusions

Within the field of spinal cord regeneration research in the urodeles, the role of ependymoglial cells, and the factors that influence their behaviours, have been extensively examined (Enos et al., 2019). They are of particular interest due to their involvement with extracellular matrix formation and removal, remodeling of radial glial processes, and associations with axonal outgrowth, stem cell properties, and neurogenesis (Egar et al., 1972, Chernoff, 1996, Zukor et al., 2011; Becker et al., 2015). Endocannabinoids are also known to respond to nervous system injury through their synthesis and release from post-synaptic dendrites and their impact on the behaviours of nearby neurons and glia through complex signaling pathways (Scheller & Kirchhoff, 2016). Prior to this thesis, data on the role of endocannabinoid signaling in spinal cord regeneration in urodeles was non-existent. In fact, the only study that has provided evidence for a role for the ECS in regeneration of any structures in urodeles was by Rao et al. (2014) where they identified the upregulation of DAGLB, the enzyme that catalyzes the conversion of DAG to 2-AG, after axolotl limb amputation. Unfortunately, this was a global proteomic analysis of the protein expression patterns that are altered after limb amputation and does not further examine the role of the ECS during this process. The authors do, however, suggest that endocannabinoids may play a role in nerve regeneration and pain control in blastema formation (Rao et al., 2014).

There are a number of studies that demonstrated a role for the ECS in response to nerve damage in simpler, invertebrate nervous systems, such as those of the nematode and leech. However, the studies that are performed in vertebrates are limited to organisms that demonstrate a limited capacity to regenerate from traumatic injuries in the spinal cord. Thus, the purpose of my thesis was to test the hypothesis that endocannabinoid signaling

plays a role in controlling the ependymoglial response and in regulating the proliferation of this NSC population after spinal cord injury/tail amputation in axolotls.

5.1. Injury-induced temporal and spatial patterns of CB1 and CB2 expression.

My original prediction was that CB1 and CB2 receptors would be expressed in ependymoglial cells based on previous studies detailing their expression on glial cells during nervous system damage in rodents (Massi et al., 2008). Furthermore, I aimed to test the hypothesis that amputation of the tail and spinal cord leads to an upregulation of cannabinoid receptor expression.

I found support for this hypothesis, as I provided evidence for both an upregulation of cannabinoid receptor expression after amputation and localization of these receptors to the ependymoglial cells (CB1) and the subependymal cells (CB2) respectively. I identified a 4-fold increase in CB1 expression compared to the uninjured tissue as early as 4 hpa which was maintained until 14 dpa. CB2 expression was not upregulated as early but was significantly increased at 7 and 14 dpa.

My findings also localized CB1 expression mainly in the ependymoglial cells, a primary NSC population (Chernoff et al., 2018). It is possible that the upregulation of CB1 expression may be required to induce proliferation of these stem cells to develop the ependymal tube. Although my present data do not directly support this, one could speculate that the early upregulation of endocannabinoid signaling in the ependymoglia may alter or inhibit gene expression patterns normally responsible for the production of a glial scar in regeneration-incompetent vertebrates such as mammals.

I also identified an injury-induced upregulation of CB2 expression within the subependymal region. This region is composed of actively dividing cells that form various

newly generated neurons, oligodendrocytes, astrocytes, and microglial cells (Zamore, 1978; Hui et al., 2010). Unfortunately, my thesis did not investigate the expression of cannabinoid receptors within microglia of the axolotl, but this represents an important area for future studies.

5.2. Active endocannabinoid signaling is required for tail and spinal cord regeneration

I next hypothesized that active endocannabinoid receptor signaling is required for tail and spinal cord regeneration in the axolotl. By employing a morphometric regeneration assay based on that of Ponomareva et al. (2015), I found support for this hypothesis. Seven days of constant bath treatment with either selective reverse agonists AM251 (CB1) or AM630 (CB2) led to impaired tail and spinal cord regeneration. Similar results were obtained with a 1-day pulse treatment with each reverse agonist to account for the potential toxic effects resulting from constant treatment. No such inhibition was found with a much shorter 4-hour pulse treatment, however. It is possible that 4 hours of reverse agonist treatment may not have been enough time to inhibit the regenerative process over the course of the 7-day regeneration period. My western blotting analyses and immunofluorescence imaging revealed that treatment with either inverse agonist resulted in a decrease in their respective CB1 or CB2 receptor expression. Surprisingly, I identified an increase in the number of GFAP+ cells within the first 4 hours of treatment, after 7 days constant treatment, and after 1-day pulse treatments using qualitative analyses of the immunofluorescence images.

My finding that downregulation of cannabinoid signaling through either CB1 or CB2 inverse agonists results in an increase in the number of cells expressing GFAP

suggests a critical role for the ECS in providing a permissive environment for regeneration. It would be interesting to determine whether there is a possible link between the trauma-induced upregulation of miR-200a and regulation of some components of the ECS pathway (Sabin et al., 2019). In addition, further analysis on whether downstream effectors of the ECS may regulate the GFAP promotor in these cells may provide more insight on this admittedly complex system.

5.3. Correlation of cannabinoid receptor expression with proliferating and differentiating cell populations

In addition, I hypothesized that ECS signaling is correlated with proliferating and differentiating cell populations within the caudal regenerating spinal cord. I provide evidence of CB1 and CB2 expression on proliferating cells from 7 dpa regenerate tissue. In particular, I found the colocalization of CB1 and BrdU+ in presumably the ependymoglial cells, based on their location along the central canal and their radial glial morphology. Alternatively, CB2 expression was occasionally colocalized with BrdU+ cells in the subependymal domains. I also provided data for the colocalization of cannabinoid receptors and DCX on cells presumably undergoing neuronal differentiation in the ependymal tube. Although CB1 and CB2 have been demonstrated to have a role during proliferation and differentiation (as mentioned in previous chapters), further studies with selective antagonists of the cannabinoid receptors are needed to determine the relative contributions of the two receptor types to the ECS-mediated regulation of neurogenesis during axolotl spinal cord regeneration.

5.4. Potential downstream effectors of injury-induced cannabinoid receptor upregulation

Lastly, I examined the potential downstream signal transduction pathways mediating the response of spinal cord cells to receptor activation. My western blotting data demonstrated a decrease in the activated pERK during the first three days post tail amputation, but these differences are diminished and not significant by day 7. This statistically significant decrease in the activated form of ERK (pERK) might be required for the initial stages of spinal cord regeneration. Given my western blotting data in Chapter 2, it is possible that this is associated with CB1 activation. Further investigation identifying potential alterations in the ERK and pERK expression patterns within regenerating animals treated with an inverse agonist of CB1 is necessary to decipher whether this downstream pathway mediates ECS signaling through CB1 activation in the early stages of spinal cord regeneration.

5.5. Conclusions and perspectives

As mentioned previously, the role of glia in response to injury when comparing CNS regeneration-incompetent and competent organisms differs drastically. However, the contributions of the ECS in glia functionality has become a promising avenue for biomedical research associated with neuropathology as various enzymes for endocannabinoid synthesis, transport, and degradation are found within glia (Luongo et al., 2010; Stella, 2004; Scheller & Kirchhof, 2016).

The original aim of this thesis was to examine the role the endocannabinoid system in regulating the ependymoglial response in the regenerating spinal cord in the axolotl. Based on my results, it is difficult to assign precise roles for ECS signaling through either

CB1 or CB2 in this process. However, I did provide evidence of temporal and spatial upregulation of these receptors during critical timepoints during regeneration, receptor expression in ependymoglial cells and subependymal cells undergoing proliferation and differentiation, and impaired tail and spinal cord regeneration after normal cannabinoid receptor signaling is disrupted.

5.6. Future directions

There are further considerations that can be made to strengthen the findings of my thesis. For a majority of my thesis, my claims are supported using western blotting and immunofluorescence applications. However, these experiments used CB1 and CB2 antibodies designed against the rat sequences rather than the axolotl. Although I have performed pre-adsorption controls testing for antibody specificity and outlined potential reasons for the molecular weight discrepancies in my western blot data from Chapter 2, experiments can be performed to assess the ability of these primary antibodies to recognize the CB1 and CB2 protein in axolotl tissue. Such an experiment would include a protein identification analysis with the assistance of Harvard's Mass Spectrometry and Proteomics Resource Lab to identify if the axolotl CB1 and CB2 are amongst the peptide fragments in the bands I identified in my western blots. Alternatively, we can construct a plasmid containing the sequences of the axolotl CB1 or CB2 under the control of a strong constitutive promoter and transfect them into a mammalian cell line in which the endogenous receptor genes have been knocked out. A western blot can then be performed on homogenates from these cells with the mammalian antibodies to determine if the bands I identified as CB1 and CB2 in my current thesis are still expressed and of similar molecular weights. Furthermore, qPCR analyses using CB1 and CB2 specific primers could be

employed to examine mRNA transcripts for these receptors to further support my findings during these timepoints.

Performing BrdU+ (proliferation) and DCX+ (differentiation) immunofluorescence analyses on tissues acquired from inverse agonist-treated animals could provide further support for a role for the ECS in regulating the decisions of the ependymoglial cells to divide or initiate the path to differentiation. These studies were, in fact, initiated but not completed due to the mandated laboratory shut-down associated with the COVID-19 pandemic. In addition, my immunofluorescence images currently rely on qualitative analyses, making it difficult to draw conclusions. Further quantitative analyses would benefit my thesis to identify significant changes in the number of cells expressing GFAP, BrdU, or DCX between different timepoints or treatments. This was original goal of my current thesis but was also unfortunately unfinished due to the restricted laboratory entries during the pandemic.

There is also evidence that the ECS can play a role later in CNS development and regeneration during the period of axonal pathfinding subsequent to neuronal differentiation. Endocannabinoids can act as guidance molecules in the developing mammalian nervous system (Fernández-Ruiz et al., 2000) and CB1 has been localized to growth cones in neuronal terminals (Katona et al., 1999). It would be interesting to see if this is true in axolotls as well.

As mentioned previously, determining the association between spinal cord injury induced miRNAs with components of the ECS would be useful. The effects of inhibiting the ECS with inverse agonists on expression of these regulatory miRNAs could provide a link between the ECS and the gene products required for initiating the ependymoglial

response. Conversely, one could also monitor the effects on ECS signaling of artificially inhibiting or upregulating the production of specific miRNAs (e.g., miR-200a) via introducing mimics or inhibitors of these miRNAs into the ependymoglial cells via *in vivo* electroporation.

Lastly, there is also the consideration of investigating these findings in other regeneration-competent vertebrates including the zebrafish or larval *Xenopus*. Indeed, these have been used as models for spinal cord regeneration; however, their use in examining the ECS has been limited to neurodevelopment. By employing these organisms, it would allow for a more comprehensive study of the role of the ECS during spinal cord regeneration in regeneration-competent vertebrates.

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Appendix

Table A1. Dose-lethality data in varying concentrations of CB1 inverse-agonist, AM251. The concentration of inverse agonists for the bathing treatments was determined by testing a variety of concentrations cited in the literature. This examined the effects of bathing larval axolotls in concentrations of AM251 ranging from 1μM to 25 μM. Over a 7 day regeneration period, it was observed that 1μM AM251 did not have a severe impact on the survival rate of the animals when compared to the higher concentrations despite impacting the animals' ability to regenerate their tail. Furthermore, animals in 1μM AM251 were able to recover after the treatment period. For these reasons, 1μM was the concentration used on my studies. These data were also used as a reference for the CB2 inverse agonist, AM630. Animals were bathed in a similar range of concentrations of the vehicle control, DMSO:Tween-80 with no measurable impact on survival rate or tail regeneration in any stage.

Treatment & Concentration	**n**	**Survival Rate (%)**	**Proportional Incr. in Body Length ± S.E**
AM251 25μM	8	0% by Day 4	NA
AM251 20μM	8	0% by Day 4	NA
AM251 10μM	8	0% by Day 5	NA
AM251 5μM	8	0% by Day 6	NA
AM251 2.5μM	8	0% by Day 7	NA
AM251 1μM	20	85%	0.0997 ± 0.032

Table A2. List of Solutions.

Solution	Directions
5x Gel Tank Denaturing Running Buffer	30.28 g Tris 144.13g Glycine 10g SDS Add MilliQ to 2L
10x Protein Transfer Buffer (PTB)	30.28g Tris 144.13 Glycine Add MilliQ to 1L
10x Phosphate Buffered Saline (PBS)	80g NaCl 2g KCl 14.2g Na_2HPO_4 2.4g KH_2PO_4 Add MilliQ to 800 mL Adjust pH to 7.4 Add MilliQ to 1L
1x Protein Transfer Buffer (PTB)	100mL 10x PTB 600mL MilliQ 200mL Methanol Add MilliQ to 1L
1x Gel Tank Denaturing Running Buffer	200mL 5x Gel Tank Denaturing Running Buffer Add MilliQ to 1L
1x Phosphate Buffered Saline (PBS)	100mL 10x PBS Add MilliQ to 1L
1x PBS/0.1%Tween-20	Make 1xPBS soln 1mL Tween-20 for 1L solution
1x PBS/3% Non fat Milk powder /0.1% Tween-20	Make 1x PBS soln 30mg of Milk powder 1mL Tween-20 for 1L solution

12% Resolving Gel (2 gels for western blot)	6.9 mL MilliQ 5.0 mL 1.5M Tris HCl (pH 8.8) 8.0 mL Bis-acrylamide 30% solution 100 µL 20% SDS 100 µL APS 10 µL TEMED
4% Stacking Gel (2 gels for western blot)	7.4 mL MilliQ 1.25 mL 1.0 M 1.0 Tris-HCl (pH 6.8) 1.3 mL Bis-acrylamide 30% solution 50 µL 20% SDS 50 µL APS 10 µL TEMED
30% Sucrose	30g sucrose into 70mL of 1xPBS Mix solution, then adjust volume to 100mL
5% Sucrose	5g sucrose into 70mL of 1xPBS Mix solution, then adjust volume to 100mL
1xPBS/0.1-0.3% Triton	Make 1xPBS 1mL of Triton for 1L solution