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The impact of morphine on nerve injury recovery and lipid metabolism pathways

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The impact of morphine on nerve injury recovery and lipid metabolism pathways

by

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Abstract

Neuropathic pain resulting from peripheral nerve injury is among the most debilitating types of chronic pain conditions. Opioid medications are often used despite their poor efficacy in treating neuropathic pain symptoms and concerns about adverse effects. Notably, emerging evidence suggests that rather than alleviating neuropathic pain, opioids may worsen mechanical allodynia, wherein innocuous stimuli elicit pain. Morphine has been shown to exacerbate nerve injury-induced mechanical allodynia, but the cause is not understood. Both morphine and nerve injury have been implicated in myelin and oligodendrocyte perturbations. As myelin is primarily composed of lipids, here I determined whether spinal lipid metabolism alterations are a potential mechanism underlying morphine exacerbated neuropathic pain. In this study, I characterized neuropathic pain development and recovery using the chronic constriction injury model (CCI), which results in robust mechanical allodynia. I demonstrated that delayed morphine treatment of CCI animals resulted in prolonged mechanical allodynia and recovery from nerve injury, as compared to nerve injured mice given saline. Using profiling arrays for cholesterol metabolism and lipoprotein signaling, I found that morphine further increases the CCI-induced lipid metabolism changes within the spinal cord. More specifically, I found that cholesterol metabolic and lipoprotein clearance pathways are upregulated in nerve injured animals given saline when earlier and later timepoints are compared, suggesting their role in recovery. Conversely, these same pathways are downregulated in morphine treated CCI animals. To confirm these results, I validated the six most dysregulated gene candidates and found that *Cyp11a1* was significantly upregulated in CCI animals treated with morphine. These results suggest that morphine could be delaying recovery from CCI by dysregulating lipid metabolism at the spinal cord level. As glial cells are involved in lipid synthesis and maintenance of myelin, alterations in lipid metabolism could be linked to changes in oligodendrocytes, astrocytes, microglia, and myelination.

These findings could help mitigate adverse opioid effects and improve treatment for people suffering from neuropathic pain.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
^{o}c	Degrees Celsius
$^{\Delta\Delta}$Ct	Delta-Delta Cycle Threshold
μ	Mu
δ	Delta
κ	Kappa
Abca1	ATP-Binding Cassette Sub-Family A Member 1
Acaa2	Acetyl-Coenzyme A Acyltransferase 2
Ankra2	Ankyrin Repeat, Family A (Rfxank-Like) 2
ANOVA	Analysis Of Variance
Apoa2	Apolipoprotein A-II
Apoa4	Apolipoprotein A-IV
Apoc3	Apolipoprotein C-III
Apoe	Apolipoprotein E
ATP	Adenosine triphosphate
BBB	Blood Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BL	Baseline
C57BL/6J	C57 Black 6 Jackson
cAMP	Cyclic Adenosine Monophosphate
CCI	Chronic Constriction Injury
Cdh13	Cadherin-13
cDNA	Complementary Deoxyribonucleic Acid
Cel	Carboxyl Ester Lipase
Cnbp	Cellular Nucleic Acid-Binding Protein
CNS	Central Nervous System
CSF-1	Colony-Stimulating Factor 1
Cxcl16	C-X-C Motif Chemokine 16
Cyb5r3	NADH-Cytochrome B5 Reductase 3
Cyp11a1	Cytochrome P450, Family 11, Subfamily A, Polypeptide 1
Cyp7b1	Cytochrome P450, Family 7, Subfamily B, Polypeptide 1
D14	Day 14
DAMPs	Danger associated molecular patterns
DAPI	4',6-Diamidino-2-Phelindole
Dhcr24	24-Dehydrocholesterol Reductase
Fdft1	Farnesyl Diphosphate Farnesyl Transferase 1
Gapdh	Glyceraldehyde-3-Phosphate Dehydrogenase
Gusb	Glucuronidase Beta
Hdlbp	High Density Lipoprotein Binding Protein
HIV	Human Immunodeficiency Virus
Hmgcs2	Hydroxymethylglutaryl-Coa Synthase
Hsp90ab1	Heat Shock Protein 90 Alpha Family Class B Member 1
i.p.	Intraperitoneal
IBA1	Ionized Calcium Binding Factor 1
IL-18	Interleukin-18

IL-1β	Interleukin-1 Beta
IL-33	Interleukin-33
Il4	Interleukin-4
Insig1	Insulin-Induced Gene 1 Protein
Insig2	Insulin-Induced Gene 2 Protein
kg	Kilogram
KOR	Kappa-Opioid Receptor
Ldlrap1	Low Density Lipoprotein Receptor Adapter Protein 1
Lrp12	Low-Density Lipoprotein Receptor Related Protein 12
Lrp1b	Low-Density Lipoprotein Receptor-Related Protein 1b
MBP	Myelin Basic Protein
mg	Milligram
MOR	Mu-Opioid Receptor
NG2	Neural/Glial Antigen 2
NIH	National Institutes of Health
NMDA	N-Methyl-D-Aspartate
Nr0b2	Nuclear Receptor Subfamily 0 Group B Member 2
OPC	Oligodendrocyte Precursor Cell
Osbpl5	Oxysterol-Binding Protein-Related Protein 5
P2X7	P2X Purinoceptor 7
PBS	Phosphate Buffered Saline
Pcsk9	Proprotein Convertase Subtilisin/Kexin Type 9
PDN	Painful Diabetic Neuropathy
PFA	Paraformaldehyde
Pmvk	Phosphomevalonate Kinase
PNS	Peripheral Nervous System
Prkaa1	5'-AMP-Activated Protein Kinase Catalytic Subunit Alpha-1
PWT	Paw Withdrawal Threshold
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
S1P	Sphingosine-1-Phosphate
S1PR1	Sphingosine-1-Phosphate Receptor Subtype-1
Scap	Sterol Regulatory Element-Binding Protein Cleavage-Activating Protein
Scarf1	Scavenger Receptor Class F Member 1
SEM	Standard Error of the Mean
Snx17	Sorting Nexin-17
SNRIs	Serotonin-Norepinephrine Reuptake Inhibitors
Soat2	Sterol O-Acyltransferase 2
ST2	Soluble Interleukin 1 Receptor-Like 1
Stab1	Stabilin 1
TBS	Tris Buffered Saline
TLR-4	Toll-Like Receptor 4
Tm7sf2	Transmembrane 7 Superfamily Member 2
TNF-α	Tumor Necrosis Factor Alpha
Vldlr	Very Low-Density Lipoprotein Receptor
W4	Week 4

Introduction

Pain is a complex sensory and emotional experience (Julius & Basbaum, 2001). Acute pain has an evolutionarily protective function to alert us to injury and cue defensive responses. However, acute pain can transition to chronic pain, which is defined by persistent or recurring pain for longer than three months (Treede et al., 2019). In Canada, it is estimated that 19% of the population live with chronic pain (Schopflocher et al., 2011). The economic burden of chronic pain in Canada was estimated to be $38.3-$40.3 billion in 2019 (Campbell et al., 2020).

Neuropathic pain is a type of chronic pain where pain arises from injury or pathology of the nervous system (Scholz et al., 2019). In Alberta, 18% of the population is impacted by neuropathic pain, which is diagnosed alongside conditions such as Multiple sclerosis and Diabetes mellitus (Toth et al., 2009). The hallmark symptoms of neuropathic pain include hyperalgesia (increased pain in response to painful stimuli), mechanical allodynia (pain in response to non-painful stimuli), and spontaneous pain (Colloca et al., 2017). The treatment options for neuropathic pain are limited and a reduction in a patient's pain by 20% is considered a success (Mu et al., 2017). Primary treatments of chronic neuropathic pain include gabapentinoids, tricyclic antidepressants and serotonin-norepinephrine reuptake inhibitors (SNRIs) (Mu et al., 2017). Despite opioids remaining the gold standard treatment for moderate to severe acute pain, opioid medications are recommended as a second line treatment for chronic neuropathic pain due to their lack of efficacy, and potential for adverse side effects and abuse.

Acute Pain

Acute pain is a result of nociceptive signaling from the site of injury in the periphery to higher brain regions. The spinal cord is a key relay station for the regulation of nociceptive information. In the periphery, noxious mechanical or chemical stimuli activate peripheral nociceptors which communicate this nociceptive information via specialized primary afferent fibers to the central nervous system (Latremoliere & Woolf, 2009). Nociceptors detect noxious stimuli in the skin, muscle, joints, and viscera (Gold & Gebhart, 2010). A unique characteristic of nociceptors is their sensitization in response to noxious stimuli, which is defined as a reduction in their threshold and an increase in their excitability (Gold & Gebhart, 2010). Nociceptors are classified into A or C fibers and are defined by their conduction velocity, which is correlated to their axon diameter and whether they are myelinated (Dubin & Patapoutian, 2010). Medium diameter, myelinated Aδ fibers effectuate fast onset pain in response to noxious stimuli, while large diameter, myelinated Aβ afferents respond to innocuous mechanical stimuli (Basbaum et al., 2009; Dubin & Patapoutian, 2010). In contrast, small diameter, unmyelinated C-fibers transmit slowly and respond to a range of thermal, mechanical, or chemical noxious stimuli (Dubin & Patapoutian, 2010).

Primary afferent neurons connect peripheral nociceptors to the spinal cord and terminate within the various laminae of the spinal dorsal horn. The superficial laminae, I and II, receive input from Aδ and C fibers, while the deeper laminae, III to VI, are innervated by Aβ fibers (Todd, 2002). Conversely, proprioceptive wide dynamic range neurons, which process input from other peripheral organs, terminate in lamina V, as well as C fibers (Basbaum et al., 2009; D'Mello & Dickenson, 2008).

Lamina I is highly populated with projection neurons and is the primary location of Aδ and C fiber synapses with ascending secondary afferents (D'Mello & Dickenson, 2008). These projection neurons form pathways such as the spinothalamic and spinoreticular tracts and are responsible for communicating nociceptive information to higher brain regions (Jessel, 1991). The pathway continues with the thalamus receiving this information and relaying it to cortical regions, forming the sensory portion of the pain experience. Beyond the nociceptive signals communicated along these pathways, pain perception can be influenced by cognitive and emotional factors (Bushnell et al., 2013). Despite the characterization of the pathways underlying pain, why acute pain can sometimes develop into chronic pain is not fully understood. In contrast to acute pain, chronic pain outlasts the normal time of healing and serves no functional purpose. A particularly debilitating type of chronic pain is neuropathic pain, which results from damage to the nervous system. As neuropathy is a highly prevalent disorder with a lack of effective treatment options, it is imperative to gain a better understanding of how chronic pain is established.

Peripheral and Central Sensitization

A crucial process underlying the switch from acute to chronic pain is sensitization. Without noxious input, peripheral nociceptors are inactive (Gold & Gebhart, 2010). In response to noxious stimuli, sensitization results in lowering the activation threshold and increasing the excitability of peripheral nociceptors (Gold & Gebhart, 2010). In contrast to acute pain which subsides, sensitization can persist and become maladaptive in chronic pain (Gold & Gebhart, 2010). Peripheral sensitization can transition from the site of injury to the central nervous system and lead to chronic pain long after the initial injury has healed. Peripheral sensitization can result in spontaneous primary afferent neuron firing, leading to hyperexcitability at the spinal cord level (Baron, 2006). It can also lead to mechanical allodynia, where input from thick, myelinated Aβ

fibers, which normally transduce mechanical stimuli such as light touch, is felt as pain instead (Baron, 2006). The hyperactivity at the primary afferent level leads to significant changes in the spinal dorsal horn and central sensitization. Central sensitization has been suggested as a pathophysiological mechanism underlying chronic neuropathic pain (Latremoliere & Woolf, 2009).

In lamina I, peripheral nociceptors and abnormally sensitized Aβ fibers communicate nociceptive information to second order neurons projecting supraspinally (Jessel, 1991). After peripheral nerve injury, sensitized primary afferents release neurotransmitters such as glutamate (Bleakman et al., 2006) and substance P (Khasabov et al., 2002) leading to spinal hyperexcitability (Baron, 2006). Neuronal excitability in second order neurons is triggered by the upregulation, trafficking, and phosphorylation of excitatory ion channels such as the N-methyl-D-aspartate (NMDA) receptor and voltage gated calcium channels (Li et al., 2004; Ultenius et al., 2006). Central sensitization can lead to increase in sensory receptive field area and response to stimulation, resulting in nociception in the absence of stimuli (Costigan et al., 2009; Latremoliere & Woolf, 2009). These central sensitization mechanisms have been established in animal models and could underlie the neuropathic pain symptoms of allodynia, hyperalgesia, and spontaneous pain in humans (Latremoliere & Woolf, 2009). The transition from peripheral sensitization to central sensitization in the establishment of neuropathic pain has traditionally focused on neuronal mechanisms. However, there is now considerable evidence showing the importance of glia in the pathoetiology of neuropathic pain (Inoue & Tsuda, 2018).

Glia and Pain

In addition to neuronal hyperexcitability and central sensitization, inflammatory signaling between neurons and glia contributes to neuropathic pain (Finnerup et al., 2021). In the CNS, attention has been focused on the involvement of microglia, astrocytes and to a lesser extent, oligodendrocytes. Microglia, the resident immune cells of the CNS, proliferate, change morphology, and become activated within the spinal dorsal horn in response to signals from injured or activated primary afferent neurons such as adenosine triphosphate (ATP) and colony-stimulating factor 1 (CSF-1) (Inoue & Tsuda, 2018; Ji et al., 2016). Activated microglia continue this neuroinflammatory cascade by releasing various cytokines and factors, such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-18 (IL-18), and brain-derived neurotrophic factor (BDNF) (Coull et al., 2005). In response to cytokine release, there is decreased conversion of excess glutamate by astrocytes, further amplifying this effect (Grace et al., 2014). This glial signaling cascade further promotes neuronal excitability and potentiates central sensitization. This is also increased by the formation of reactive oxygen species during neuropathic pain states, which sustains glutamatergic signaling (Squillace & Salvemini, 2022).

Although less is known about oligodendrocytes, the myelinating glia of the CNS, their high levels of metabolic activity make them susceptible to apoptosis due to the pro-inflammatory factors and oxidative stress in this milieu (Thorburne & Juurlink, 2002; Xu et al., 2004). One study shows that oligodendrocyte precursor cell (OPC) proliferation markedly increases in the ipsilateral spinal dorsal horn after peripheral nerve injury (Echeverry et al., 2008). On a molecular level, interleukin-33 (IL-33) produced by oligodendrocytes is upregulated in the dorsal spinal cord and the absence of IL-33 receptor, ST2, results in lower levels of pain in mice (Zarpelon et al., 2016). Furthermore, many painful pathologies result from disruption to oligodendrocytes and myelin. In Multiple

sclerosis, where there is autoimmune mediated damage to myelin and oligodendrocytes, pain is a prevalent symptom (Solaro et al., 2004). This led to studies investigating the relationship between loss of oligodendrocytes and pain symptoms, which found that elimination of oligodendrocytes results in pain independent of the immune system (Gritsch et al., 2014).

In a rat model of Painful Diabetic Neuropathy (PDN), the number of myelinated fibers in the spinothalamic tract and oligodendrocytes in the spinal dorsal horn is increased (J. Lin et al., 2020). Although peripheral nerve demyelination is a signature of PDN, the authors propose that the heightened numbers of oligodendrocytes in the spinal cord are a result of maladaptive plasticity and overcorrection to the demyelination in the periphery (J. Lin et al., 2020). The number of spinal oligodendrocyte precursor cells are also increased in human immunodeficiency virus (HIV) patients with chronic pain, further indicating a role of oligodendrocytes in chronification of pain (Shi et al., 2016). Collectively, this demonstrates the important role of glia in the cellular landscape of neuropathic pain and highlights the need to consider the interactions between all cell types within the CNS, especially when developing new treatments for pain.

Glia, Pain, and Opioids

Opioids are among the most widely used pain medications. Although opioids are not considered highly effective for treating neuropathic pain, they are considered the second line of treatment and often prescribed to people with this condition (Hoffman et al., 2017). For example, a regional study in the United States showed that opioid medications were prescribed to 69.4% of peripheral neuropathy patients, with 18.8% receiving chronic opioid therapy (Hoffman et al., 2017).

The spinal mechanisms of action of opioids have been well established within the dorsal horn. This region is key for the communication of nociceptive information to the brain and for the

analgesic action of opioids (Fields, 2004). Opioids exert their analgesic effects by acting on μ, δ, κ opioid receptors, resulting in suppressed spinal nociceptive transmission (Trang et al., 2015). Opioid receptors within the spinal dorsal horn are mainly localized in the first and second laminae (Stevens et al., 1991) on presynaptic primary afferents (Lamotte et al., 1976), interneurons (Kemp et al., 1996), and postsynaptic second order neurons (Zieglgänsberger & Bayerl, 1976). On presynaptic primary afferents, opioids decrease nociceptive transmitter release by reducing cAMP signaling and voltage-gated calcium channel activity, which also suppresses nociceptive signals from ascending (Trang et al., 2015). Opioids also induce hyperpolarization of post-synaptic second order neurons by activating G protein-coupled inwardly-rectifying potassium channels (Trang et al., 2015). Opioids also activate descending inhibition of the dorsal horn from the periaqueductal gray and rostral ventromedial medulla, resulting in analgesia (Trang et al., 2015). Despite the efficacy of opioids in the treatment of acute pain, their negative side effects, potential for abuse and tolerance are causes for concern.

Microglia, astrocytes, and opioids

In addition to their actions on neurons, opioids impact the activity of glia through opioid receptors. Microglia express μ (Machelska & Celik, 2020), κ (Missig et al., 2022) and δ (Shrivastava et al., 2017) opioid receptors. These same receptors are also expressed on astrocytes (Eriksson et al., 1993; Machelska & Celik, 2020; Reiss et al., 2021). In addition, opioids can bind the toll-like receptor 4 (TLR4), which are primarily expressed on microglia, but also are present on astrocytes within the CNS (Green et al., 2022). Furthermore, a multitude of studies have demonstrated the involvement of glia in the negative side effects that accompany opioids (Roeckel et al., 2016). Glia have been implicated in the mechanism of several opioid-related side effects such as repeated opioid administration leading to analgesic tolerance, opioid-induced persistence of pain, and

opioid-induced hyperalgesia (Roeckel et al., 2016). The glial mechanisms underlying these side effects include increased glial inflammatory cytokine and chemokine release and oxidative stress within the spinal cord (Grace et al., 2015; Salvemini & Neumann, 2009). Animal model studies have shown that antagonizing these targets can decrease the negative side effects of opioids, providing a promising direction for exploring glial specific pain therapeutic targets (Roeckel et al., 2016). An interesting recent study described how both opioids and neuropathic pain states altered sphingolipid metabolism in the CNS (Doyle et al., 2020). Repeated opioid administration increased sphingosine-1-phosphate (S1P) neuroinflammatory signaling between microglia and astrocytes (Doyle et al., 2020). This study demonstrated how targeting the S1P receptor subtype 1 (S1PR1) prevented the development of opioid tolerance without impacting the analgesic effect of morphine and blocked the effect of morphine prolonging neuropathic pain (Doyle et al., 2020). Moreover, this study presented new avenues of treating neuropathic pain and decreasing the negative side effects of opioid treatment via targeting lipid metabolism. There has been additional evidence underlying the link between neuropathy, lipids, and glia. An exciting study found that neuroinflammatory receptors within the membrane of spinal microglia were anchored within cholesterol-enriched lipid rafts (Navia-Pelaez et al., 2021). The administration of apoA-I binding protein reversed neuropathic pain by decreasing cholesterol from inflammarafts and buildup of lipid droplets within microglia, thus halting neuroinflammatory signal propagation (Navia-Pelaez et al., 2021). Together, these studies demonstrate the dysregulation in lipid metabolism as a therapeutic target for neuropathic pain and the importance of further investigating how lipids are altered in neuropathic pain states. However, these studies focused on microglia and astrocytes, even though oligodendrocytes also have opioid receptors (Knapp et al., 1998) and are sensitive to the neuroinflammatory environment that can accompany opioid administration.

Oligodendrocytes and opioids

Initial studies focusing on the relationship between the endogenous opioid system and oligodendrocytes found that oligodendrocytes express different opioid receptors at different stages in maturation. Mu-opioid receptors (MORs) were expressed early in oligodendrocyte development, while kappa-opioid receptors (KORs) were detected in more mature oligodendrocytes (Knapp et al., 1998). Immature oligodendrocytes responded to MOR agonists by increasing proliferation while there was no effect in mature oligodendrocytes (Knapp et al., 1998). Conversely, when KORs were antagonized, there was an increase in oligodendrocyte differentiation *in vitro* (Knapp et al., 1998). In subsequent experiments, the expression of endogenous opioids by oligodendrocytes were found to be regulated based on maturation state (Knapp et al., 2001) It was also discovered that KOR antagonist administration increased oligodendrocyte death. (Knapp et al., 2001). Based on these findings, the authors proposed that oligodendrocyte survival and development are modulated by endogenous opioids via autocrine and/or paracrine mechanisms (Knapp et al., 2001). Due to the relationship between the endogenous opioid system and remyelination, studies have also explored KORs as a potential avenue for remyelinating therapies. (Du et al., 2016; Mei et al., 2016). Another group then showed that after treatment with the MOR antagonist naloxone, oligodendrogenesis in naïve cultures decreased by 50% (Persson et al., 2003). These studies demonstrate the importance of the endogenous opioid system for oligodendrocyte maturation and myelination and how disruption of these system with opioid medications could be perturbing crucial brain connections and processes (Knapp et al., 1998; Vestal-Laborde et al., 2014).

Given the developmentally regulated expression of oligodendrocyte opioid receptors, subsequent studies investigated the effects of opioid administration at pre- and post-natal stages of nervous

system development (Sanchez et al., 2008). Prenatal exposure to a clinically relevant and overexposure dose of buprenorphine, a partial MOR agonist and KOR antagonist, had dose and time-specific impacts on pups (Sanchez et al., 2008). In a dose of buprenorphine clinically relevant to that prescribed to pregnant women with opioid addictions, there was increased levels of myelin basic protein (MBP), a myelin marker. However, in a higher, overexposure level dose, the expression of MBP was decreased until later timepoints in development (Sanchez et al., 2008). In corpus callosum axons, buprenorphine treatment increased the number of large caliber axons, however decreased their myelin thickness (Sanchez et al., 2008). Another maintenance therapy for people with opioid addictions, methadone, has been shown to impact myelination in rat pups (Vestal-Laborde et al., 2014). Methadone increased the number of corpus callosum axons with mature myelin *in vivo* (Vestal-Laborde et al., 2014). In cultured cells, methadone increased the proliferation of OPCs and the maturation of preoligodendrocytes (Vestal-Laborde et al., 2014).

Emerging evidence suggests a link between opioid exposure and altered oligodendrocyte function, but the mechanism and consequence of this interaction are not well defined. RNA sequencing has uncovered an oligodendrocyte-specific transcriptional response to morphine, which proposes a mechanism for the opioid abuse correlated white matter alterations (Avey et al., 2018). Another molecular mechanism has recently been discovered where morphine administration increased the levels of cytokine IL-33 in oligodendrocytes and the corresponding receptor, ST2, in astrocytes (Hu et al., 2021). Inhibition of IL-33 signaling reduces opioid-induced hyperalgesia (Hu et al., 2021). The implication of oligodendrocytes in morphine administered for chronic pain provides an avenue to explore other oligodendrocyte-related pathways that may be involved. The changes observed in white matter after opioid use suggest that opioids could be disrupting oligodendroglial lipids responsible for maintaining myelin, which requires further investigation.

Lipids and Myelin

The central nervous system is rich in lipids, which play a variety of crucial roles. Lipids not only support the structure of neural membranes but also assist signal transduction across membranes and anchoring of other proteins within biomembranes (Agranoff BW et al., 1999). The brain is enriched in cholesterol, containing 23% of the body's total cholesterol (Dietschy & Turley, 2004). Most of the brain's cholesterol is used to create myelin, the multilayered insulator that is important for the efficient conduction of action potentials.

In the central nervous system, myelination is dictated by oligodendrocytes, whereas in the peripheral nervous system (PNS), Schwann cells have this distinction. CNS myelination is a dynamic and ongoing process, however PNS myelination does not continue into adulthood (Poitelon et al., 2020). While a single oligodendrocyte can myelinate multiple myelin sheath segments for different neurons, a Schwann cell myelinates only the axon segment of a single neuron (Poitelon et al., 2020).

Oligodendrocytes not only provide insulation, but also support important metabolic processes for myelinated axons (Moore et al., 2020; Nave & Werner, 2014). Oligodendroglia are comprised of many different maturation states: oligodendrocyte precursor cells (OPCs), pre-oligodendrocytes, immature oligodendrocytes, and myelinating mature oligodendrocytes (Marinelli et al., 2016). Conversely, Schwann cells can differentiate into myelinating or non-myelinating.

Oligodendrocytes are responsible for the synthesis of the fatty acid component of myelin (Dimas et al., 2019). Another major component of myelin is cholesterol, which cannot cross the blood brain barrier (BBB) and therefore must be synthesized in the CNS (Björkhem & Meaney, 2004). As the peripheral nerves lack a BBB, Schwann cells can take up cholesterol from the circulation, as well as synthesize cholesterol *de novo* (Goodrum et al., 2000; Jurevics & Morell, 1994). In the

CNS, oligodendrocytes and neurons are the primary producers of cholesterol during development (Marangon et al., 2020).

During adulthood, the responsibility of producing cholesterol shifts to astrocytes, who distribute it through apolipoproteins to neurons and oligodendrocytes (Camargo et al., 2017; Marangon et al., 2020). However, when lipid synthesis by astrocytes is blocked, oligodendrocytes compensate by inserting surrounding lipids into myelin (Camargo et al., 2017). If cholesterol synthesis by oligodendrocytes is blocked, this disrupts and reduces the rate of myelination (Saher et al., 2005). In addition, microglia have recently been discovered to play a role in regulating myelin level homeostasis (McNamara et al., 2023). Overall, lipids are critical for proper nervous system function. Disruption of lipid homeostasis impacts the function of glia and the essential processes they support, such as myelination.

There is a gap in our understanding of how glia and myelin are affected by morphine treatment of neuropathic pain. By studying how lipids relevant for myelin are impacted within the spinal cord, we can better understand how both morphine and nerve injury are leading to dysregulated lipid metabolism. **I hypothesize that morphine treatment alters lipid metabolism within the spinal cord and underlies the delay in recovery from nerve injury.**

Research Statement

Emerging evidence suggests that rather than alleviating neuropathic pain, opioids may worsen pain symptoms. Morphine has been shown to exacerbate mechanical allodynia caused by nerve injury, but the cause is not yet understood. Lipid signaling and metabolism are critical processes within the central nervous system, especially in the production and maintenance of myelin. Despite the essential role of myelin for proper nervous system function, the impact of morphine on lipids following peripheral nerve injury has yet to be determined. Here, I investigate how lipid metabolism is dysregulated in morphine exacerbated neuropathic pain. Below, I will provide an overview of the key objectives and hypothesis that were tested in mice.

Research Objectives

Aim 1: Determine the impact of morphine treatment on recovery from peripheral nerve injury.

CCI is known to produce robust mechanical allodynia with recovery ranging from 6-8 weeks after surgery. Previous groups have demonstrated the effect of morphine prolonging CCI recovery in different strains of male rats using the von Frey Filament test (Grace et al., 2016). I tested the reproducibility of these observations and determine the timeline of progression and recovery in male mice, using both evoked (von Frey) and non-evoked (Dynamic Weight Bearing) tests. After demonstrating a timeline of CCI progression and recovery, I administered morphine ten days after CCI, when mechanical allodynia had been established. I compared when saline versus morphine treated CCI animals recovered to determine the impact and timeline of morphine prolonging recovery from nerve injury.

Aim 2: Determine the effects of peripheral nerve injury and morphine on lipid metabolism.

To investigate what is underlying delayed recovery in morphine treated CCI animals, I evaluated transcriptional changes within the spinal cord. This unbiased screening approach identified candidates within important pathways. As cholesterol is the primary lipid component of myelin, alterations in lipoprotein signaling and cholesterol metabolism could suggest how myelin is impacted in this context. Thus, RNA profiling arrays with primers specific to these pathways were used to identify candidate genes relevant to lipid metabolism within the spinal cord. To understand how lipids are involved in delayed recovery, spinal cord tissue was collected at the previously determined timepoint when saline treated CCI animals had recovered, but the morphine treated animals had not. To determine the acute effects of morphine on nerve injury, spinal cord tissue was also collected after the five days of morphine administration. In addition, comparing the same groups across time allows insight into impacted pathways relevant for recovery from CCI. I have further validated these changes through confirmation quantitative polymerase chain reactions (qPCRs) with primers specific for the six most dysregulated candidate genes. These experiments revealed multiple candidate genes involved in pathways important for the production and maintenance of myelin.

Overall, the identification of lipid-related candidate genes provides new avenues for teasing apart the consequences of delayed morphine treatment of neuropathic pain on myelination and oligodendrocytes. Understanding the perturbations of myelin-relevant lipids underlying morphine treatment could help with the development of more effective therapies for neuropathic pain.

General Methodology

Animals and Housing

Adult male C57BL/6J mice were obtained from Jackson Laboratories and housed in groups of four under a 12-hour light/dark cycle with *ad libitum* access to food and water. All animal procedures were approved by the University of Calgary Animal Care Committee (Protocol AC20-0131) and are in accordance with the guidelines of the Canadian Council on Animal Care.

Chronic Constriction Injury (CCI) Model of Neuropathic Pain

Chronic constriction injury (CCI) was used to study peripheral nerve injury induced neuropathic pain. In this model, three loose 7-0 prolene sutures (Ethicon) were tied around the left sciatic nerve of ten-week-old male C57BL/6J mice. The CCI model recapitulates the mechanical allodynia observed in neuropathic pain patients. It is well characterized, the resulting pain phenotype is robust, and the animals show recovery (Austin et al., 2012). Sham animals underwent an identical surgery, but without the three sutures around the sciatic nerve.

Behavioral testing and morphine treatment paradigm

To assess mechanical allodynia in nerve injured animals, I performed the von Frey filament test and the dynamic weight bearing test. The von Frey filament test was performed three times in the week before the CCI to establish a baseline paw withdrawal threshold, and after CCI on days 1, 3, 7, 10, 12, 14, and weekly thereafter. The paw withdrawal threshold was determined based on the up-down method (Chaplan et al., 1994). On each day of habituation and testing, animals are habituated to the testing apparatus for at least 30 minutes. To assess non-evoked neuropathic pain, the dynamic weight bearing test was administered twice in the week before the surgery, after the surgery on days 2, 4, 6, 11, 13, 15, and weekly afterwards (Sheehan et al., 2021). Animals were

briefly habituated to the dynamic weight bearing testing apparatus before three minutes of acquisition. From the three minutes acquired, a 30 second portion was validated manually. Morphine sulfate (pentahydrate) was injected intraperitoneally (i.p.) once daily from days 10 to 14 after CCI at 10 mg/kg. Control animals were given an equivalent volume of saline daily for seven days.

Immunohistochemistry

Mice were anesthetized with isoflurane, perfused transcardially with PBS, and then 4% (w/v) paraformaldehyde (PFA) in 0.1M phosphate buffered saline. Spinal cords were isolated by hydraulic extrusion, post-fixed in 4% PFA, cryoprotected in 30% sucrose and stored at 4ºC. Lumbar spinal cords were sliced into 40 µm free-floating sections, blocked for one hour in 0.3% Triton X-100 in TBS with 5% Normal Donkey Serum, then incubated overnight in primary antibodies (Goat IBA1 1:500; Rabbit NG2 1:500 (Millipore AB5320)). Sections were washed and incubated with fluorophore-conjugated secondary antibodies (Donkey anti-Goat AlexaFluor 647 1:500, Donkey anti-Rabbit AlexaFluor 488 1:500) for two hours. Before mounting, sections were washed again and incubated with DAPI (1:10000, Abcam AB228549) on the final wash. Images were obtained using the VS110 5-Slide Scanner and quantification was performed using ImageJ (NIH).

RNA Extraction

Samples for RNA work were generated from morphine or saline treated CCI or sham animals from the 14 days post CCI and 4 weeks post injection timepoints. Mice were anesthetized with isoflurane, then perfused transcardially with PBS to remove blood. Spinal cords were isolated by hydraulic extrusion, flash frozen in liquid nitrogen and stored at -80ºC. The lumbar region of the spinal cords was isolated and homogenized. RNA extraction was performed according to the

manufacturer's protocol for TRIzol (ThermoFisher). Lumbar spinal cord sections homogenized with 1mL of TRIzol were shaken with 200µL of chloroform and incubated at room temperature for 5 minutes for phase separation. The aqueous layer was transferred to new tube, added to 500 µL of isopropanol and placed on ice for 10 minutes. RNA was pelleted by refrigerated centrifugation (12000g for 10 minutes), washed with 75% ethanol and centrifuged again (7500g for 5 minutes). The RNA pellet was air dried and resuspended in 50 µL of UltraPure distilled water (Catalog 10977015, Thermofisher) before incubation at 65 degrees for 5 minutes. RNA was quantified using a NanoPhotometer (Implen, NP80-Mobile) and stored at -80ºC.

RNA Profiling Arrays

For the profiling arrays, the three samples with the highest RNA quality were chosen from each of the four experimental groups from both timepoints with four animals per group (24 samples total). The RT2 First Strand Kit (Catalog 330404, Qiagen) was used generate cDNA from 0.5µg of isolated RNA, which was stored at -20ºC. RT2 Profiler PCR Array Format C were chosen according to the thermocycler and contained 84 primers for Mouse Cholesterol Metabolism and Lipoprotein Signaling, as well as 5 housekeeping genes, and controls for genomic DNA, reverse-transcription, and positive PCR (Catalog 330231, Qiagen). CDNA was subjected to quantitative qPCR using RT2 SYBR Green ROX qPCR Master Mix (Catalog 330523, Qiagen) on the Applied Biosystems Quant Studio 3 Thermocycler according to the Qiagen RT2 Profiler Handbook. Results were analyzed using the Qiagen Gene Globe RT2 Profiler PCR Data Analysis Software according to the RT2 Profiler PCR Array Data Analysis Handbook.

Validation qPCR

RNA extracted from all four animals in the morphine and saline treated CCI groups at both timepoints (14 days post CCI and 4 weeks post injection) was used to validate the RNA profiling

array candidates (16 samples total). CDNA was synthesized using VILO SuperScript Master Mix (Catalog 11755050, Thermo Fisher) using 1µg of isolated RNA and stored at -20ºC. Pre-designed primers were obtained for the six selected candidate genes (*Apoe (*Mm.PT.58.33516165), *Dhcr24 (*Mm.PT.58.5182147), *Pcsk9* (Mm.PT.58.5401743*), Soat2* (Mm.PT.58.6537447), *Cyp11al* (Mm.PT.58.14210705), and *Abca1* (Mm.PT.58.9651201) (Integrated DNA Technologies). From the 5 housekeeping genes used in the RNA profiling arrays, the gene with the most consistent gene expression across groups were selected for validation via qPCR (*Hsp90ab1* (Mm.PT.58.43472263.g), Integrated DNA Technologies). The qPCR plates were run using SYBR Green Supermix (Catalog 1725275, BIO RAD) on the Applied Biosystems Quant Studio 3 Thermocycler and analyzed using the $^{\Delta\Delta}$CT method with normalization to the housekeeping gene (*Hsp90ab1*) and the Ct values from saline treated animals 14 days post CCI were used as a control.

Statistics

All data are presented as the mean ± standard error of the mean (SEM). Statistical analyses for behavioural experiments were performed with GraphPad Prism 9 software using two-way ANOVA with *post hoc* Šídák multiple comparison test. For the qPCR profiling arrays, statistics were performed by the Qiagen Gene Globe software, which stated: "the p-value is calculated based on a Student's t-test of the replicate $2^{-\Delta CT}$ values for each gene in each Control Group and test Group comparison and is based on a parametric, unpaired, two-sample equal variance, two-tailed distribution". The $^{\Delta\Delta}$CT values from the validation qPCRs were analysed using a two-way ANOVA with *post hoc* Tukey multiple comparison test.

Results

CCI injury induces mechanical allodynia

The CCI is a model of neuropathy that mimics nerve compression injuries seen in humans. It results in the progression of mechanical allodynia, one of the most common symptoms of neuropathic pain, and is uniquely able to model recovery. To establish a recovery model of neuropathic pain, CCI or sham surgeries were performed on adult male mice and the progression of their mechanical allodynia and recovery were followed for nine weeks using the von Frey filament test (Figure 1) and Dynamic Weight Bearing test (Figure 2).

Peripheral nerve injury caused by CCI resulted in robust mechanical allodynia, which resolved seven weeks after injury (Figure 1). Paw withdrawal threshold was significantly lower in nerve injured animals than non-injured sham animals from days 1 to 21 post-CCI and then increased from day 21 (week three) until recovery at day 49 (week seven) (Figures 1 and 2). As control, the paw withdrawal threshold of the contralateral paw was measured and was not significantly different between sham and nerve injured animals (Figure 1B).

To verify the recovery timeline from CCI, dynamic weight bearing was used to evaluate non-evoked neuropathic pain (Figure 2). Mice displayed significant weight-bearing asymmetry, shown by reduced weight bearing on the ipsilateral compared to the contralateral hind paw (Figure 2A). In addition, the surface area of the affected paw was decreased (Figure 2B).

At the recovery timepoint 63 days after CCI, when mechanical threshold returned to preinjury baseline levels, I found that DAPI, NG2 and IBA1 immunostaining was comparable in both sham control and CCI mice (Figure 3). Future experiments will assess earlier timepoints as expression of these cellular markers may have been altered following nerve injury but have returned to baseline upon recovery.

Overall, the CCI method of peripheral nerve injury is sufficient to induce mechanical allodynia, from which animals recover after seven weeks. These findings provide evidence that the CCI surgery was consistently performed and provide a timeline for establishment of mechanical allodynia (day 10) and recovery (day 49). The timeline of mechanical allodynia progression informed the chosen timepoint for morphine administration in further experiments. Immunohistochemistry results were expected since the mice had recovered from the CCI at this point. Overall, this experiment characterized the surgical model using two behavioral methods which showed parallel seven-week recovery timelines.

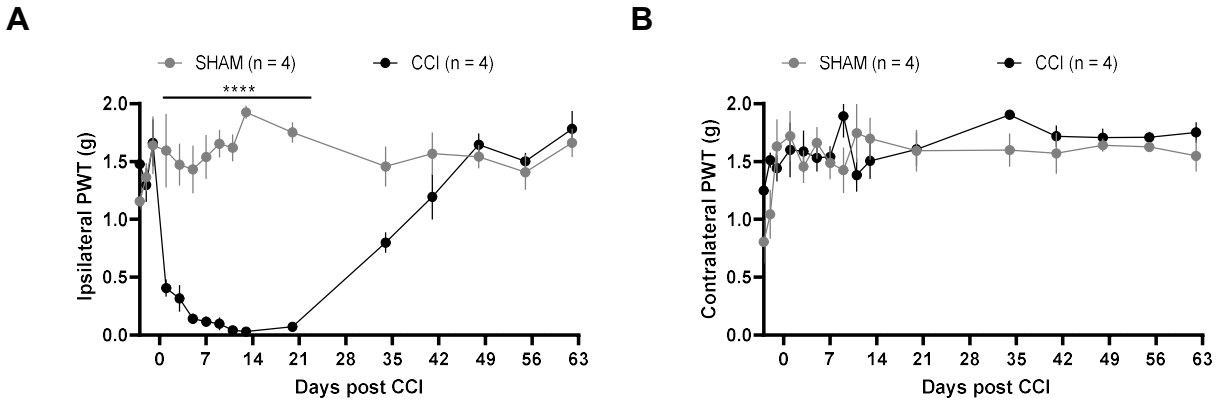

Figure 1. Time course of CCI induced mechanical allodynia.

Time course of CCI induced mechanical allodynia assessed by Von Frey filaments on the ipsilateral (A) and contralateral (B) paw of male mice. Results are mean ± SEM and analyzed by two-way ANOVA with the Šídák multiple comparisons test (****P < 0.0001).

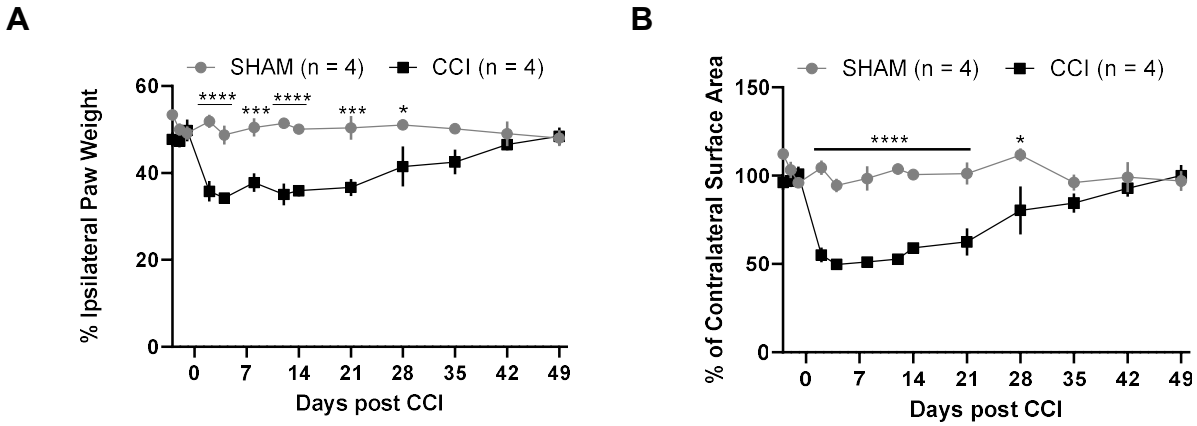

Figure 2. CCI leads to uneven paw weight and surface area distribution.

Time course of non-evoked neuropathic pain in CCI and Sham mice assessed by the Dynamic Weight Bearing test. (A) % ipsilateral paw weight is the ipsilateral paw weight percentage of total paw weight. (B) % of contralateral paw surface area is the ipsilateral paw weight percentage of contralateral paw surface area. Results are mean ± SEM and analyzed by two-way ANOVA with the Šídák multiple comparisons test (*p<0.05, ***p<0.001, ****p < 0.0001).

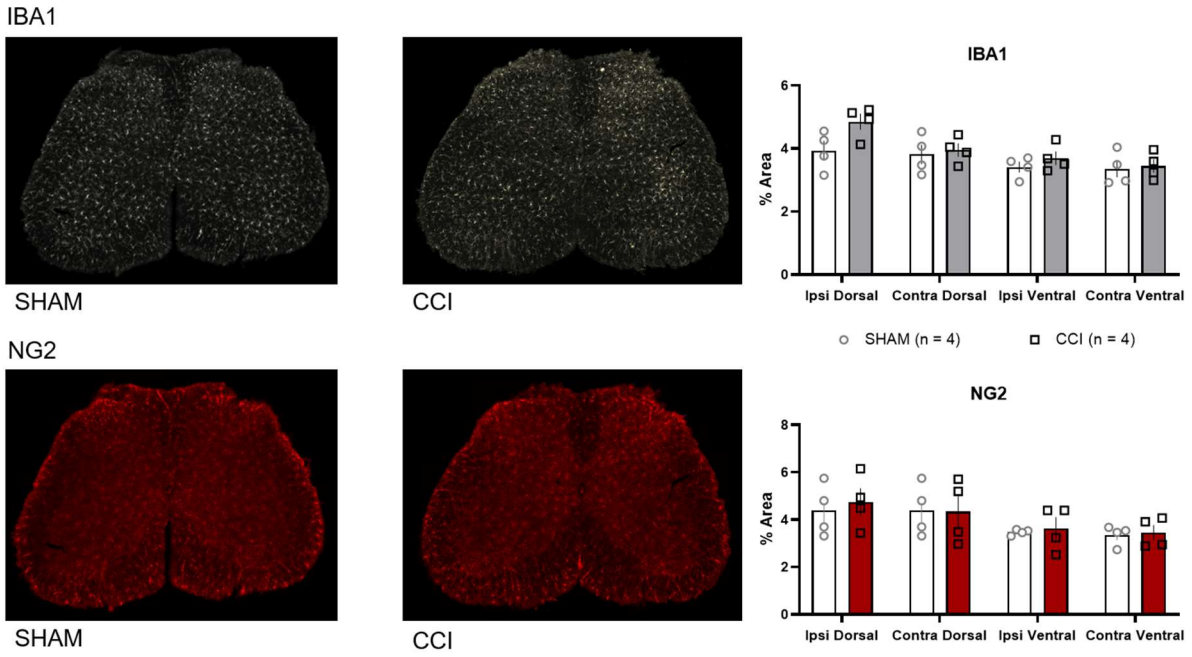

Figure 3. CCI recovered and sham animals have a comparable percentage area of microglia and OPCs.

Cross sections of CCI 63 days post recovery and sham mouse spinal cords with microglia and OPC staining (IBA1 and NG2, respectively). IBA1 and NG2 percent area from 20X images were quantified (n = 4 slices, n =4 animals). No differences were statistically significant when results were analyzed using a two-way ANOVA with the Šídák multiple comparison test.

Morphine induces anti-allodynia after CCI nerve injury

Opioids are often used to manage chronic pain conditions. Although opioids are not the first line of treatment for neuropathic pain, they are still widely prescribed and used for this condition, despite their poor efficacy. People struggling with neuropathic pain may be prescribed gabapentin, pregabalin, or SNRIs as first line treatments (Mu et al., 2017). Opioids may also be prescribed as a second- or third-line treatment, but there is often a delay from time of injury, development of neuropathic pain, and opioid treatment. This study incorporated this clinically relevant delay in opioid administration by waiting until mechanical allodynia has developed to administer morphine.

Morphine was administered to mice with established mechanical allodynia on days 10-14 post-CCI. I confirmed that an injection of morphine (10 mg/kg; i.p.) on day 10 post injury produced an antinociceptive effect. However, after 5 days of morphine treatment, the same dose of morphine on day 14 post-CCI did not affect mechanical threshold, indicating the development of morphine antinociceptive tolerance. The antinociceptive effect on the first day of morphine treatment highlights the utility of morphine in an acute setting, however the development of tolerance after five days demonstrates the decreased efficacy of repeated use.

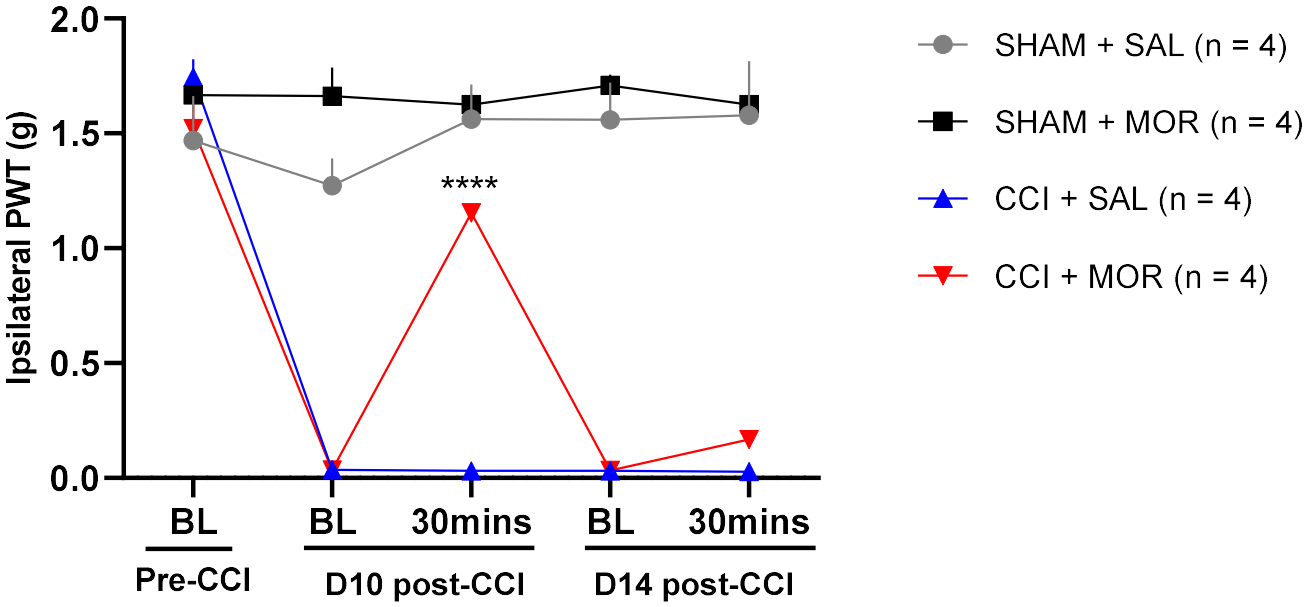

Figure 4. Acute effects of morphine on paw withdrawal threshold of CCI mice.

Von Frey filaments were used to assess mechanical allodynia at baseline, pre- and 30 minutes post-morphine (10 mg/kg) or saline injection. Morphine (10mg/kg, i.p.) or an equivalent volume of saline was administered once daily for five days to CCI or sham injured male mice. Results are mean ± SEM and analyzed by two-way ANOVA with the Šídák multiple comparisons test (****$p < 0.0001$ for CCI + MOR vs. CCI + SAL).

Repeated systemic morphine delays recovery from CCI

To determine the impact of morphine treatment on recovery from CCI, adult mice were treated with morphine (10 mg/kg, i.p., once daily) for five consecutive days, on days 10 to 14 after CCI when mechanical allodynia is well established (Figure 5). Control animals were injected with an equivalent volume of saline for five consecutive days. Mechanical paw withdrawal threshold was determined using the von Frey filament test at days one and three after CCI and weeks one through eight post morphine injection.

After nerve injured mice were given morphine or saline, there was no significant difference between groups until three weeks post injection. From the three to seven weeks post injection timepoint, there was a significant difference between the paw withdrawal thresholds of saline and morphine treated animals with nerve injury. Nerve injured animals given saline returned to their baseline paw withdrawal threshold by six weeks post injection, whereas morphine treated animals did not recover until eight weeks post injection. Therefore, morphine treatment significantly delayed the recovery from CCI-induced mechanical allodynia. By contrast, morphine treatment did not impact recovery of weight bearing asymmetry (Figure 6).

Overall, morphine treatment of nerve injury results in delayed recovery when mechanical allodynia is measured by evoked behavioural testing (von Frey filament test). However, when using non-evoked testing, I did not find a difference between morphine and saline treated weight bearing recovery from CCI nerve injury.

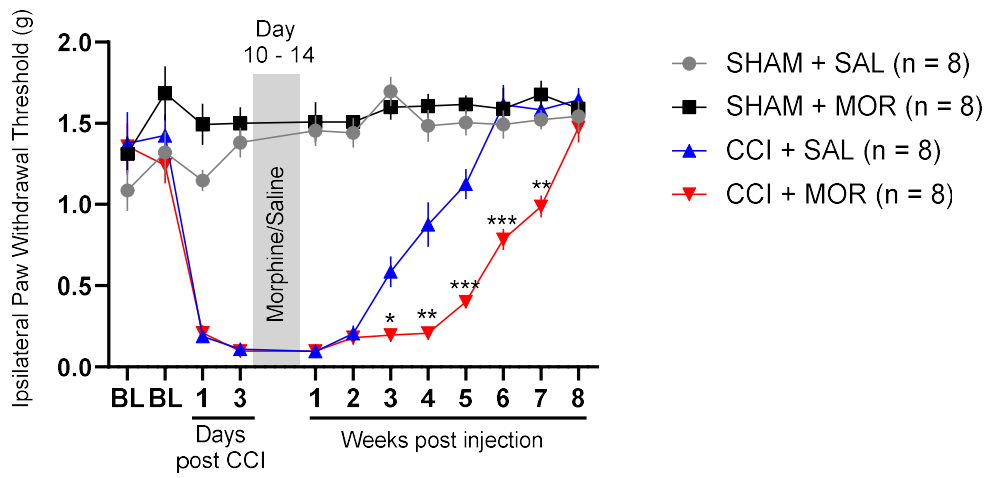

Figure 5. Morphine prolongs recovery from CCI.

Progression of CCI induced mechanical allodynia assessed by Von Frey filaments on the ipsilateral paw of male mice. Animals were treated with morphine (10 mg/kg) or saline from days 10 to 14 post CCI. Results are mean ± SEM and analyzed by two-way ANOVA with the Šídák multiple comparisons test of CCI + MOR vs. CCI + SAL (*p<0.05, **p<0.01, ***p<0.001).

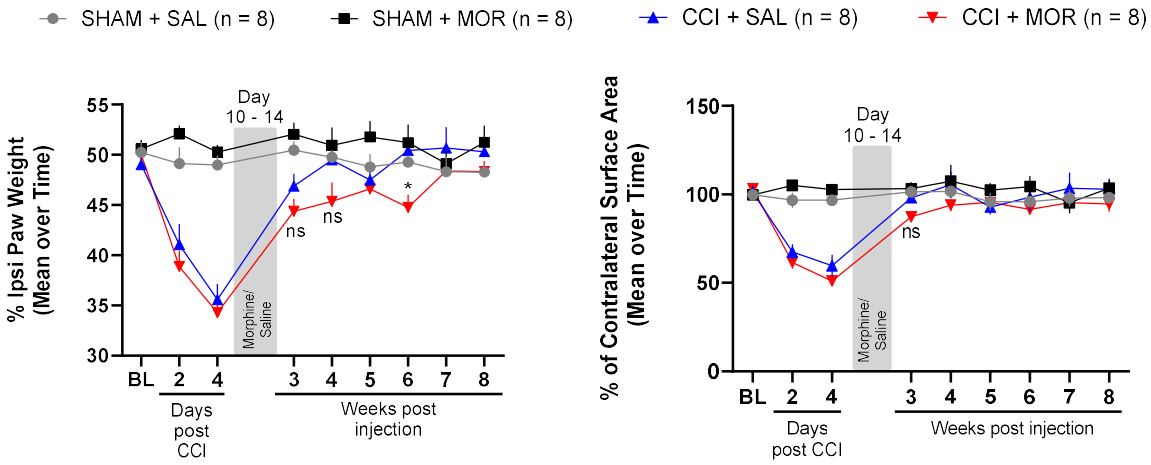

Figure 6. Morphine does not prolong CCI weight bearing recovery.

Time course of non-evoked neuropathic pain in CCI and Sham animals assessed by the Dynamic Weight Bearing test. On the tenth day after CCI, morphine (10mg/kg, i.p.) or saline was administered daily for five days. % ipsilateral paw weight is the ipsilateral paw weight percentage of total paw weight. % of contralateral paw surface area is the ipsilateral paw weight percentage of contralateral paw surface area. Results are mean ± SEM and analyzed by two-way ANOVA with the Šídák multiple comparisons test of CCI + MOR vs. CCI + SAL (*p<0.05).

Cholesterol metabolic and lipoprotein clearance pathways are dysregulated after morphine treatment of CCI

Previous studies have implicated specific bioactive lipids, astrocytes, and microglia in the prolonging of neuropathic pain by morphine (Doyle et al., 2020). Despite their focus on lipids, they did not further investigate the implications this context would have on myelin, the lipid rich, insulating layer of the nervous system. Cholesterol is highly abundant in the brain and an essential component of myelin (Dietschy & Turley, 2004). Therefore, to screen for changes in lipids within the lumbar spinal cord, I performed qPCR profiling arrays with primers specific for cholesterol metabolism and lipoprotein signaling.

To evaluate transcript-level changes after morphine treatment of CCI, RNA was extracted from the lumbar region of spinal cords of mice treated with saline or morphine after CCI or sham surgery. To compare molecular changes over time, spinal cords were collected after the last morphine injection (day 14) and once the saline treated CCI animals began to recover, but when the morphine treated animals had not (week 4) (Figure 5). The three highest quality RNA samples from each group at each timepoint were selected for cDNA synthesis and qPCR profiling arrays (Table 1).

Table 1. Quantification of spinal cord RNA quantity and quality.

RNA was extracted from lumbar spinal cords of mice at two timepoints that underwent CCI or Sham surgery and were treated for five days with either morphine (10 mg/kg) or saline, 10 days after surgery. Samples with the highest quality RNA ($A_{260/280}$ closest to 2) were chosen for qPCR profiling arrays.

		Day 14 post CCI RNA			**Week 4 post injection RNA**		
	Sample	Concentration (µg/uL)	$A_{260/280}$	$A_{260/230}$	Concentration (µg/uL)	$A_{260/280}$	$A_{260/230}$
SHAM + SAL	1	581.9	2.031	1.048	657.8	1.965	0.859
	2	676	2.023	0.885	706.55	1.896	0.47
	3	766.1	2.027	0.952	556.15	1.958	0.739
	4	642.45	2.042	1.531	1158.6	2.036	0.923
SHAM + MOR	1	878.9	2.068	1.424	212.95	1.826	0.366
	2	792.3	2.055	1.189	954.25	1.983	0.739
	3	368.1	1.919	0.498	871.25	1.978	0.712
	4	725.5	2.052	1.165	622.35	1.927	0.567
CCI + SAL	1	1054.9	2.012	0.814	832.65	2.078	1.423
	2	1104.1	1.991	0.622	983.25	2.072	1.286
	3	1018.3	1.961	0.532	816.45	2.066	1.453
	4	1093.9	1.98	0.584	903.1	2.084	1.702
CCI + MOR	1	1195.1	1.993	0.599	652.8	2.045	1.39
	2	924.6	2.031	1.027	876.7	2.082	1.854
	3	1119.8	2.048	0.785	248.35	1.972	0.941
	4	940.9	2.014	0.731	676.2	2.037	1.48

QPCR Profiling arrays

The qPCR profiling arrays revealed differentially regulated genes across all conditions. The dysregulated genes are displayed using volcano plots to visually depict candidates with a statistically significant fold change (Figure 8). To understand the changes happening in each condition over the course of recovery, I compared morphine and saline treatment of nerve injured animals immediately after the last injection (day 14) to during recovery (week 4) (Figure 7).

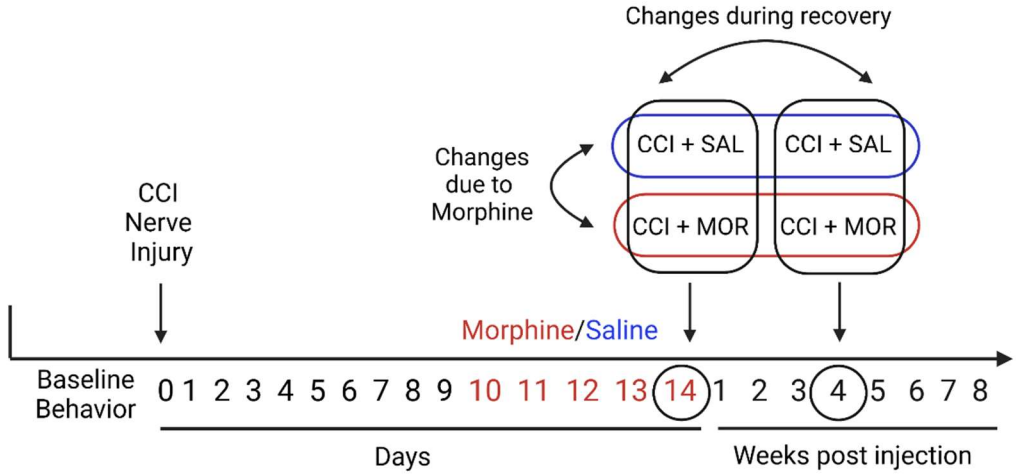

Figure 7. Timeline and schematic of nerve injury, injections, tissue collection and group comparisons in downstream analyses.

Male mice were treated for five days with either morphine (10 mg/kg) or saline, 10 days after CCI or Sham nerve injury. Tissue for RNA extraction was collected on the last day of injections (day 14) and 4 weeks later when nerve injured animals given saline had recovered but those given morphine had not.

When saline-treated control animals with nerve injury were compared directly after injection (day 14) to four weeks later, there was an upregulation in *Ankra2, Hdlbp, Pcks9, Soat2,* and *Vldlr*, whereas *Cyb5r3, Fdft1, Insig1, Gapdh* were downregulated (Figure 8). When comparing nerve injured animals shortly after morphine treatment (day 14) versus during recovery (week 4),

I found an upregulation of 2 genes (Cyp11a1, Il4) and the downregulation of 6 genes (Abca1, Cxcl16, Cyp7b1, Nr0b2, Prkaa1, Scarf1) (Figure 8).

To determine the effect of morphine at each timepoint, I then compared morphine to saline treated nerve injured animals after injection and during recovery (Figure 7). After acute treatment of morphine compared to saline in nerve injured animals, there was an upregulation of most genes (*Apoa2, Apoa4, Apoc4, Cel, Cxcl16, Pcsk9, Pmvk, Scap, Stab1*), while one was downregulated (Hmgcs2) (Figure 8). In contrast, at week 4 post injection, morphine versus saline treatment of nerve injury resulted in the downregulation of all statistically significant genes (*Acaa2, Apoe, Cdh13, Cnbp, Cxcl16, Cyb5r3, Dhcr24, Hdlbp, Insig2, Ldlrap1, Lrp12, Lrp1b, Osbpl5, Snx17, Tm7sf2*) (Figure 8). To give functional and biological relevance to these differentially regulated clusters of genes, I next performed a pathway analysis using String (Szklarczyk et al., 2021).

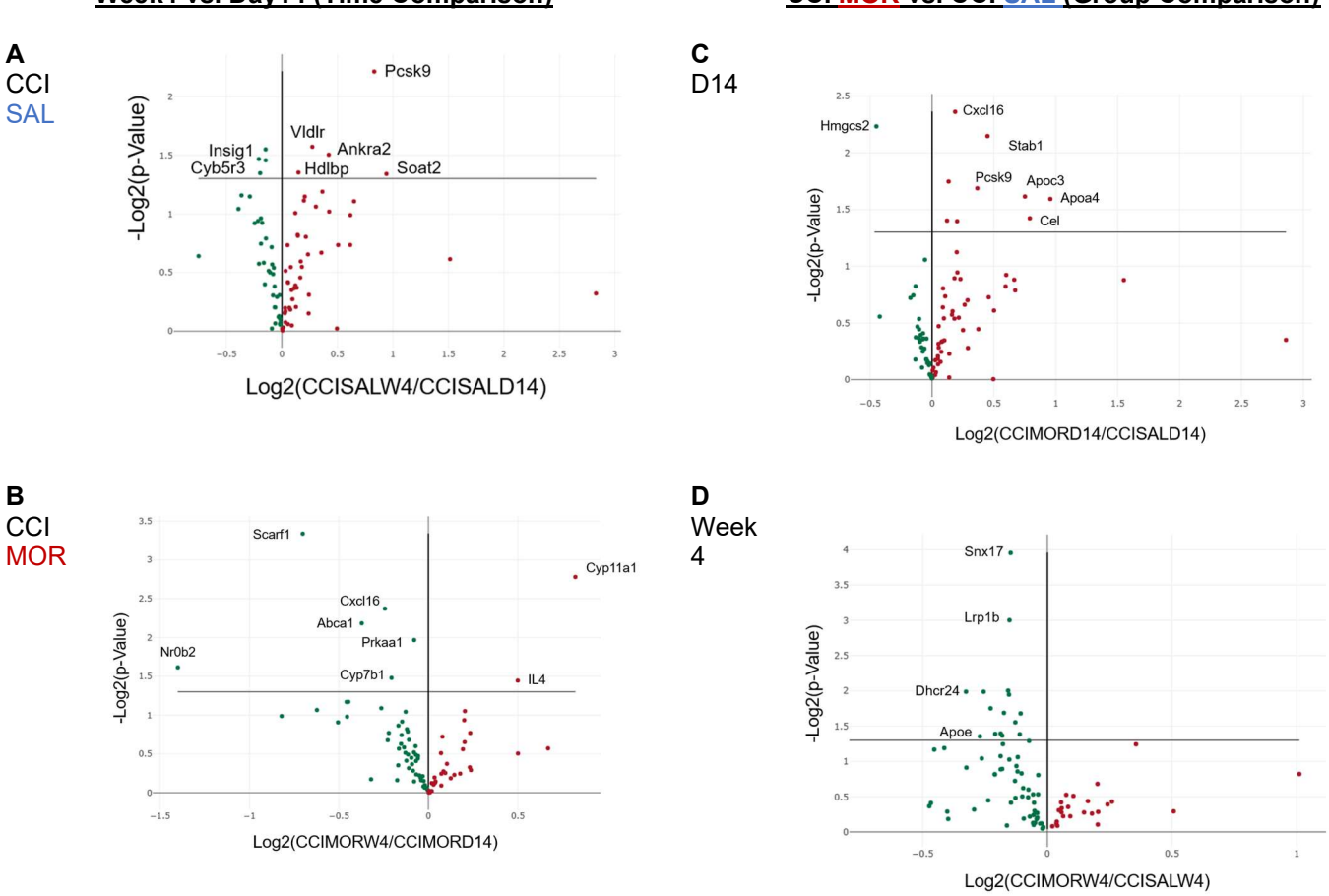

Figure 8. RNA profiling array revealed differentially regulated genes.

Mice were treated for five days with either morphine (10mg/kg; i.p.) or saline, 10 days after sham or CCI surgery. Qiagen profiling arrays were performed with lumbar spinal cord RNA isolated either 4 weeks after the last injection, or 14 days after CCI or sham surgery. Comparisons in gene expression were made between samples collected at Week 4 versus Day 14 in (A) nerve injured animals given saline and (B) nerve injured animals given morphine. Nerve injured animals given morphine were compared to nerve injured saline controls at (C) Day 14 and (D) Week 4. Volcano plots show statistically significant (above horizontal threshold line) upregulated (red) and downregulated genes (green). Each plot is the comparison of the average of gene expression from 3 RNA samples to 3 comparison samples from each group at each timepoint (24 samples total, n = 3 in each group).

Pathway Analysis

Using String Pathway analysis (Szklarczyk et al., 2021), I identified common pathways that were dysregulated to get a better understanding of the changes across each group and timepoint (Table 2). As this tool identifies many functional enrichment categories, I focused on results from two sources: Biological processes (Gene Ontology) and Reactome pathways. The tabulation of these results revealed patterns and similarities across all groups (Table 2). The most conserved biological process was the cholesterol metabolic process, while plasma lipoprotein clearance was the most common Reactome pathway (Table 2). It should be noted that morphine treatment of CCI in the comparison across day 14 and week 4 yielded no results for Reactome pathways. However, all other group comparison results included plasma lipoprotein clearance.

Next, I used String to create networks to visualize how the candidate genes interacted with each other, while highlighting the most common processes and pathways (Figure 9).

To understand how the expression of lipid-related genes change during "normal" recovery from nerve injury, I compared samples collected after injection (day 14) to four weeks later. I found that most dysregulated genes were upregulated and were involved in the cholesterol metabolic process, as well as the plasma lipoprotein clearance reactome pathway (Figure 9). Four upregulated genes take part in both pathways: *Soat2, Pcsk9, Vldlr,* and *Hdlbp* (Figure 9).

The impact of morphine treatment of CCI across timepoints was determined by the comparison between samples collected acutely after morphine treatment (day 14) to during recovery (week 4). From the common pathways identified, the genes from this comparison were only involved in the cholesterol metabolic process (Figure 9). Although *Cyp11a1* and *Il4* were upregulated, *Abca1, Cyb7b1* and *Prkaa1* were all downregulated (Figure 9).

The acute effect of morphine treatment on gene expression was deduced through the comparison of nerve injured animals given morphine versus saline controls, both from day 14. The pathway in common with the other groups was the plasma lipoprotein clearance Reactome pathway and all genes relevant to this pathway were upregulated: *Pcsk9, Apoc3, Apoa2,* and *Apoa4* (Figure 9). The dysregulated genes from this comparison were also involved in the cholesterol metabolic process; *Apoa2, Apoa4, Apoc4, Cel, Pcsk9, Pmvk,* and *Scap* were all upregulated, while *Hmgcs2* was downregulated (Figure 9).

To elucidate the impact of morphine treatment on CCI recovery, I compared the morphine to saline treated CCI animals, both from 4 weeks post injection timepoint, when saline treated, animals had started to recover but morphine treated animals had not. Interestingly, all of the dysregulated genes were downregulated and were involved in the same pathways as the dysregulated genes involved in saline treated CCI recovery (Figure 9). Both pathways converge in three of the downregulated genes: *Apoe, Hdlbp,* and *Ldlrap1* (Figure 9).

Together, these results demonstrate that plasma lipoprotein clearance is commonly upregulated in the recovery of nerve injured animals given saline and in the acute morphine treatment of nerve injury. Conversely, cholesterol metabolic processes are downregulated during the recovery of nerve injured animals given morphine.

Table 2. String Pathway Analysis identified common differentially regulated pathways. QPCR profiling arrays of RNA from morphine and saline CCI animals resulted in dysregulated genes. String Pathway Analysis was used to determine relevant pathways, from which two categories were chosen: Biological Processes and Reactome Pathways. Common pathways across groups are shown in bold.

	Across Timepoint Comparison **W4 vs. D14**	**Within Group Comparison** **CCI MOR vs. CCI SAL**
	CCI + SAL	**Day 14**
Biological Processes	**Cholesterol biosynthetic process** Cholesterol homeostasis **Cholesterol metabolic process** Organic cyclic compound metabolic process Small molecule biosynthetic process Small molecule metabolic process	Negative regulation of low-density lipoprotein particle clearance High-density lipoprotein particle assembly Triglyceride-rich lipoprotein particle remodeling Regulation of intestinal cholesterol absorption Positive regulation of cholesterol esterification Phospholipid efflux Negative regulation of cholesterol transport Reverse Cholesterol transport **Cholesterol biosynthetic process** Cholesterol efflux **Cholesterol metabolic process**
Reactome Pathways	LDL clearance **Plasma lipoprotein clearance** VLDLR internalisation and degradation	Chylomicron remodeling Chylomicron assembly Retinoid metabolism and transport **Plasma lipoprotein assembly, remodeling and clearance** G alpha (i) signaling events
	CCI + MOR	**Week 4**
Biological Processes	**Cholesterol metabolic process** Steroid biosynthetic process Lipid homeostasis Cellular response to lipid	Amyloid precursor protein metabolic process **Cholesterol biosynthetic process** **Cholesterol metabolic process** Sterol transport Receptor Mediate Endocytosis
Reactome Pathways	(No results)	Chylomicron clearance **Plasma lipoprotein clearance** Cholesterol biosynthesis Metabolism

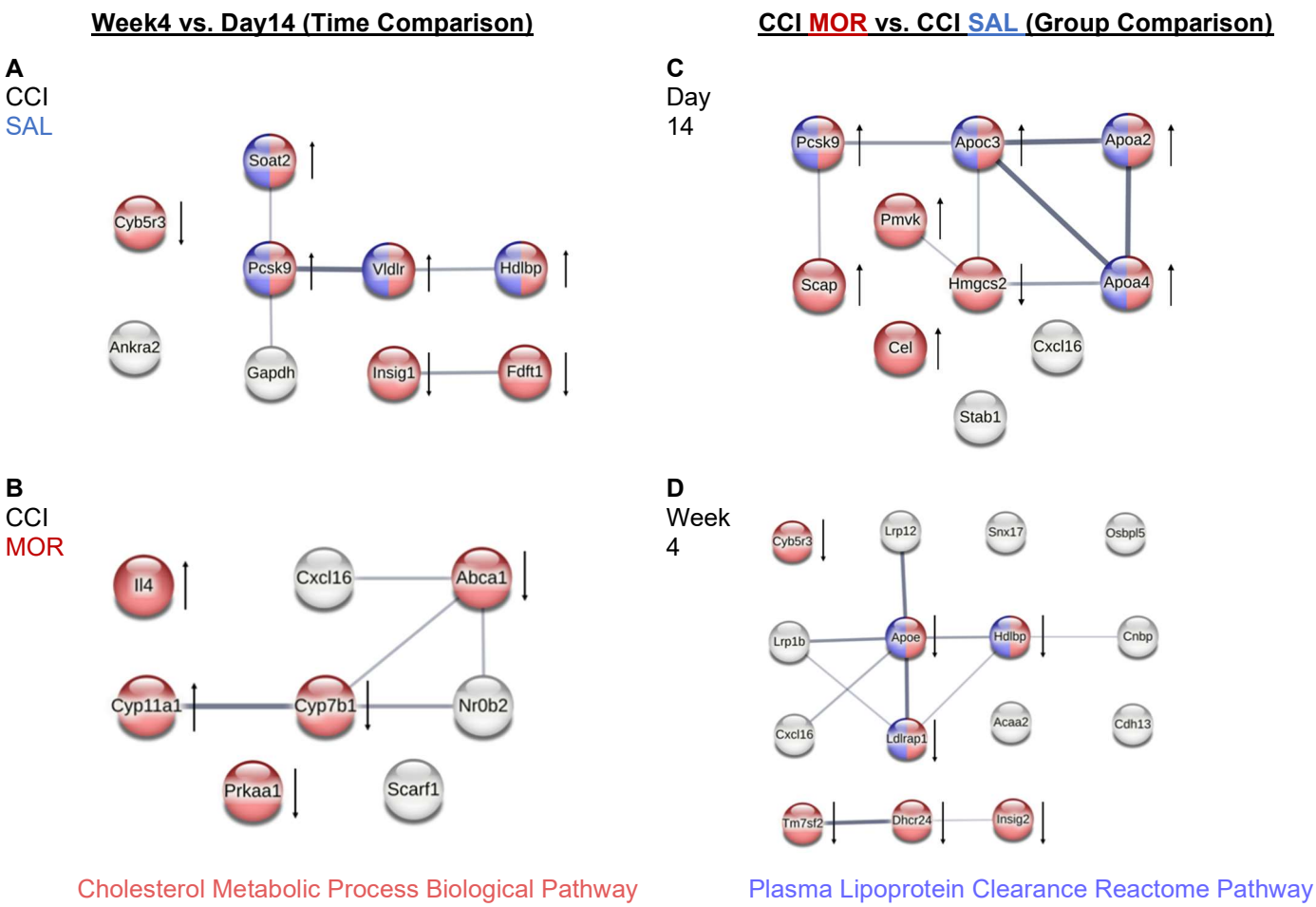

Figure 9. String Pathway Analysis of RNA profiling array dysregulated genes uncovered changes in cholesterol metabolism and lipoprotein clearance pathways.

Mice were treated for five days with either morphine (10mg/kg; i.p.) or saline, 10 days after sham or CCI surgery. Qiagen profiling arrays were performed with lumbar spinal cord RNA isolated either 4 weeks after the last injection, or 14 days after CCI or sham surgery. Comparisons in gene expression were made between samples collected at Week 4 versus Day 14 in (A) nerve injured animals given saline and (B) nerve injured animals given morphine. Nerve injured animals given morphine were compared to nerve injured saline controls at (C) Day 14 and (D) Week 4. String Pathway Analysis plots demonstrated the relationships between the protein products of the significantly up or downregulated genes, with thicker connecting lines indicating strong associations. String also identified two common pathways: cholesterol metabolic processes biological pathway (red) and plasma lipoprotein clearance Reactome pathway (purple).

QPCR validation

Having identified putative dysregulated genes from the profiling arrays, I proceeded to validate 6 of the 16 genes that showed a statistically significant fold change of 1.2 or higher, indicating a 20% change in expression (Table 3). Specifically, I selected genes with the highest fold changes in samples from nerve injured animals that were given either saline or morphine and collected at either day 14 or week 4 (*Apoe, Dhcr24, Pcsk9, Soat2).* I chose two candidates from the comparison of morphine treatment across recovery: one upregulated (*Cyp11a1*) and one downregulated (*Abca1*). qPCR was performed to validate the six candidate genes using samples from nerve injured animals given morphine or saline collected after injection (day 14) or four weeks later (Figure 10). Of the six candidate genes, only *Cyp11a1* showed significant changes in gene expression (Figure 10). The *Cyp11a1* gene was upregulated in nerve injured animals four weeks after morphine when compared to immediately after morphine injection. In addition, *Cyp11a1* expression was increased in the comparison of nerve injured animals given morphine versus saline four weeks after injection, showing upregulation across time and treatment group (Figure 10).

Table 3. Top 16 highly expressed genes from qPCR profiling arrays.

Comparison of gene expression profiles of morphine or saline treated CCI animals, 14 days post CCI or 4 weeks post injection. 16 genes were above the ±1.2 fold regulation value threshold. Six of the most differentially regulated candidates from each group comparison were selected for further validation (in bold).

	W4 CCI Mor vs SAL		**D14 CCI Mor vs SAL**		**CCI Sal W4 vs D14**		**CCI Mor W4 vs D14**	
Gene Symbol	Fold Regulation	P-value	Fold Regulation	P-value	Fold Regulation	P-value	Fold Regulation	P-value
Apoe	-1.21	0.044101						
Dhcr24	-1.25	0.010281						
Apoa4			1.94	0.025492				
Apoc3			1.68	0.024259				
Cel			1.73	0.037720				
Pcsk9			1.29	0.020516	1.78	0.006124		
Stab1			1.36	0.007107				
Hmgcs2			-1.37	0.005838				
Ankra2					1.34	0.031318		
Soat2					1.92	0.045673		
Vldlr					1.21	0.026815		
Cyp11a1							1.77	0.001663
Il4							1.41	0.035977
Abca1							-1.29	0.006570
Nr0b2							-2.64	0.024302
Scarf1							-1.63	0.000460

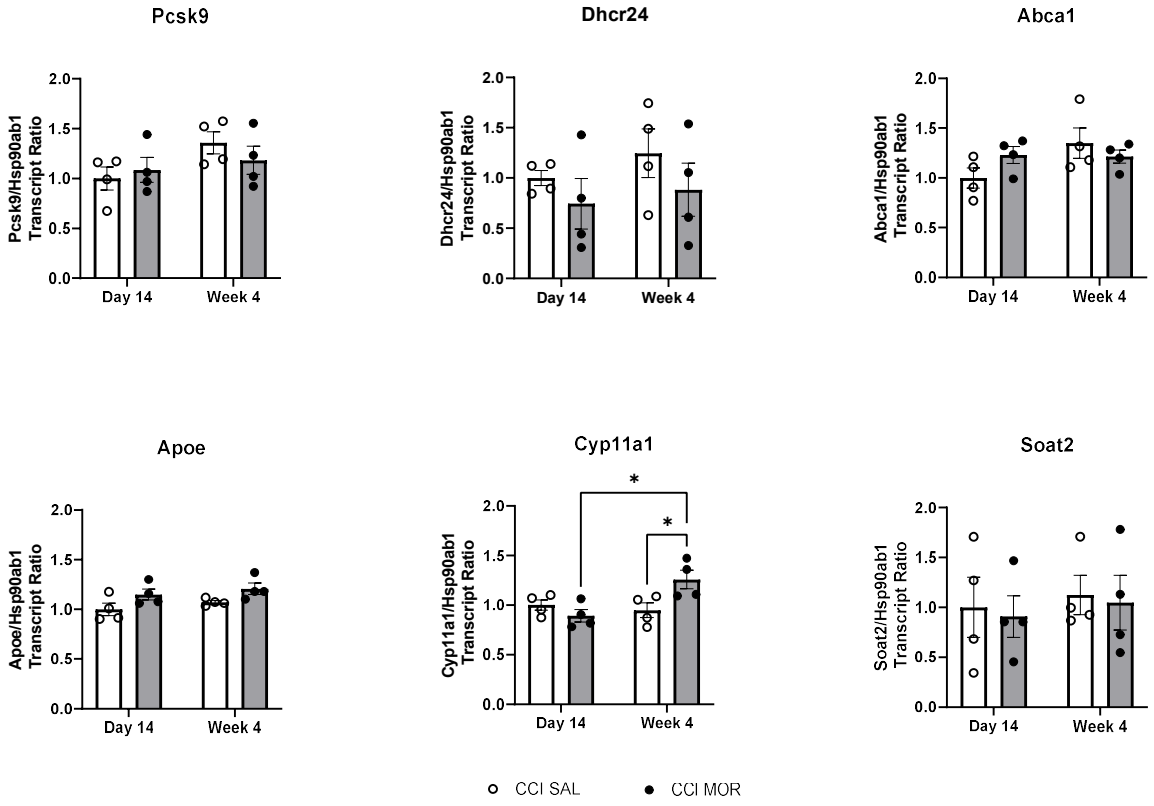

Figure 10. Gene expression profiles for validation of the six candidate genes.

Validation qPCRs for *Apoe, Dhcr24, Pcsk9, Soat2, Cyp11al,* and *Abca1* were performed using RNA isolated from morphine or saline treated CCI animals, either 14 days post CCI or 4 weeks post injection. The housekeeping gene used was *Hsp90ab1* and fold expression values were obtained using the $^{\Delta\Delta}$CT method with normalization to the D14 saline treated CCI group . Results are mean ± SEM and analyzed by a two-way ANOVA and post hoc Tukey comparison (*$p<0.05$; n=4 animals in each group).

Discussion

Summary of findings

In this thesis, I found that morphine treatment delays recovery of CCI-induced neuropathic pain and disrupts lipid metabolic and signaling pathways. Within the lumbar spinal cord, I compared gene expression changes after morphine versus saline treatment of nerve injury at two different timepoints and identified differences in converging pathways within groups and across timepoints. More specifically, I discovered significantly dysregulated gene candidates involved in cholesterol and lipoprotein clearance pathways. I confirmed that morphine treatment in nerve injured mice increased *Cyp11al* gene expression. Overall, this work indicates that peripheral nerve injury and subsequent morphine treatment causes changes in lipid metabolism which may affect the resolution of neuropathic pain.

CCI recovery

CCI is a clinically relevant neuropathic pain model as it closely recapitulates human compression injuries and conditions, such as carpal tunnel and sciatica (Padua et al., 2016; Ropper & Zafonte, 2015). In mice, a mild constriction of the nerve initially causes inflammation; however, after several weeks this eventually subsides and allows recovery from nerve injury. In humans, there is greater variability in the degree of nerve injury and recovery is dependent on the severity of the nerve injury (MacKay et al., 2021). Often repair of nerve injury is required, and functional recovery is limited (Höke, 2006). In compressive neuropathy, surgical decompression is considered the gold standard treatment but in a range of 1% to 32% of patients, symptoms persist after this intervention (Abzug et al., 2012). The failure rate of surgical decompression can partially be attributed to delayed diagnosis and the establishment of neuropathological changes in the PNS, such as intraneural scarring (Spielman et al., 2020). Nonetheless, the rodent CCI model is a useful

tool for studying recovery. I confirmed that CCI results in robust mechanical allodynia, from which animals recover weeks later, in both non-evoked and evoked neuropathic pain behaviour tests. By using a neuropathic pain model that recovered, I have tested how administration of morphine impacted recovery.

Morphine delayed recovery

Opioids have limited efficacy in relieving neuropathic pain and may even paradoxically exacerbate this pain and delay recovery (Grace et al., 2016, 2018; Green-Fulgham et al., 2019). Glial priming has been suggested as the underlying mechanism, which involves two "hits". The first "hit" arises from nerve injury which initiates an immune response, and a second "hit" caused by exposure to morphine exacerbates the inflammatory response (Grace et al., 2016). Interestingly, this paradoxical phenomenon is only observed when morphine administration is delayed, as morphine treatment at the time of nerve injury does not delay recovery (Green-Fulgham et al., 2019). The consequence of delayed opioid treatment was investigated in mice given a five-day course of morphine, ten days after CCI-induced nerve injury. This clinically relevant delay in opioid administration was incorporated because chronic neuropathic pain patients often are not treated with opioids until their pain has established, which can be months after the initial injury.

When measuring mechanical allodynia, I observed a delayed recovery due to morphine, consistent with findings reported by previous groups (Doyle et al., 2020; Grace et al., 2016). However, this effect of morphine was not observed in weight bearing recovery. Although this is the first time that dynamic weight bearing has been used to assess the effect of morphine on CCI recovery, another study using a non-evoked test has made similar observations. A study using voluntary wheel running as a measure of recovery found no prolonging of recovery in morphine treated compared to saline treated CCI animals (Green-Fulgham et al., 2022). However, when TLR4 and

P2X7 antagonists were administered alongside morphine in CCI animals, running distance and speed of CCI animals returned to sham levels (Green-Fulgham et al., 2022).

The mechanism underlying morphine delayed recovery from CCI has been investigated by several groups. The first studies to examine this phenomenon found that opioid administration of established neuropathic pain is mediated by DAMPS (Danger Associated Molecular Patterns) activating microglia via TLR4 and P2X7, leading to NLRP3 inflammasome activation within the spinal dorsal horn (Grace et al., 2016, 2018). This has been confirmed through systemic deliveries of P2X7 and TLR4 antagonists, suggesting that morphine could be acting not only at the spinal cord, but also at the sciatic nerve and dorsal root ganglion level (Green-Fulgham et al., 2022). Although μ opioid receptors have been shown to underpin detrimental side effects by opioids (Corder et al., 2017), it has been established that morphine prolongs pain after nerve injury via a non-classical opioid receptor, TLR4 (Grace et al., 2016). This work was complemented by a recent study examining through a similar mechanism how S1P, a bioactive sphingosine metabolite, is not only involved in morphine-prolonged neuropathic pain recovery, but also morphine induced hyperalgesia and tolerance (Doyle et al., 2020). They proposed that morphine induced S1P signaling, which in turn activates astrocytes and microglia via their S1PR1 receptors, increasing neuroinflammatory signaling (Doyle et al., 2020).

Morphine and CCI induced dysregulation in lipid-relevant gene expression

In this thesis, I identified dysregulated gene expression within the spinal cord in the context of morphine prolonged neuropathic pain. I made comparisons across multiple groups to ascertain how gene expression is changing over the course of recovery from nerve injury and during morphine-delayed recovery. From the 42 statistically significant dysregulated genes depicted by volcano plots, I investigated the functional relevance of these genes using String Pathway

Analysis. I chose two categories of pathways to focus this search: Biological processes and Reactome pathways. Across the Biological process results, I found that all comparisons across groups are involved in the cholesterol metabolic process, whereas in the Reactome pathways, plasma lipoprotein clearance was identified in three of the four group comparisons.

Gene expression changes in the spinal cord following nerve injury and recovery

First, I investigated the recovery process following nerve injury in control animals administered saline by comparing gene expression after injection (day 14) versus during w 4 weeks later during recovery. I found that during recovery, there is an upregulation of five genes (*Ankra2, Hdlbp, Pcks9, Soat2, Vldlr*) and a downregulation in four genes (*Cyb5r3, Fdft1, Insig1, Gapdh*). From the pathway analysis, four of the upregulated genes are involved in both the cholesterol metabolic process and plasma lipoprotein clearance pathway (*Hdlbp, Pcks9, Soat2, Vldlr*). This increased gene expression implies that upregulation in these pathways and genes may be associated with recovery from nerve injury.

In contrast, nerve injured mice exposed to morphine displayed a profile of gene changes that were distinct from nerve injured control mice given saline. Specifically, morphine treated mice showed an increase in *Cyp11a1* and *Il4* expression, and a decrease in *Abca1, Cyb7b1*, *Prkaa1, Cxcl16, Cyp7b1, Nr0b2,* and *Scarf1*. The String pathway analysis only identified the cholesterol metabolic pathway as a common pathway. Only two of the genes involved in this pathway were upregulated (*Cyp11a1* and *Il4*), while the other three were downregulated (*Abca1, Cyb7b1*, *Prkaa1*). Therefore, morphine treatment during the recovery process after nerve injury could decrease the expression of genes involved in the cholesterol metabolic pathway.

Timepoint-specific gene expression changes after morphine treatment of nerve injury

To understand the impact of morphine at distinct timepoints, I compared gene expression of nerve injured mice treated with morphine to controls given saline at day 14 and week 4. At the timepoint immediately after the five-day course of morphine treatment (day 14), I found that there was increased gene expression in nine genes (*Apoa2, Apoa4, Apoc4, Cel, Cxcl16, Pcsk9, Pmvk, Scap, Stab1*) and decreased gene expression in one (*Hmgcs2*), relative to nerve injured control animals given saline. Seven of the upregulated genes (*Apoa2, Apoa4, Apoc4, Cel, Pcsk9, Pmvk, Scap*) and the one downregulated gene (*Hmgcs2*) were involved in the cholesterol metabolic process, while all genes in the plasma lipoprotein pathway were upregulated (*Pcks9, Apoc3, Apoa2, Apoa4*). This demonstrates that acute morphine treatment of nerve injury could increase gene expression in these two pathways.

In contrast to the upregulation immediately after morphine treatment of nerve injury, gene expression 4 weeks after morphine treatment of nerve injury is decreased in all 15 genes, relative to nerve injured controls at this timepoint. All seven genes involved in the cholesterol metabolic process (*Apoe, Ldlrap1, Hdlbp, Dhcr24, Tm7sf2, Insig2, Cyb5r3*) and all three genes that play a role in the plasma lipoprotein clearance pathway (*Apoe, Hdlbp, Ldlrap1*) are downregulated. This overall gene downregulation observed in nerve injured mice treated with morphine relative to controls given saline at week 4 is distinct from the upregulations in these same pathways across other timepoints and comparisons groups.

We could broadly interpret these results by suggesting that recovery from CCI involves upregulation in cholesterol metabolic and lipoprotein clearance pathways. However, morphine administration downregulated these pathways during nerve injury recovery, which may indicate

that downregulation in cholesterol metabolic and lipoprotein clearance pathways is involved in delayed recovery from nerve injury.

Validation of lipid-relevant gene candidates

To choose candidates for further validation experiments, I determined that 16 of the 42 dysregulated genes were above a 1.2-fold regulation threshold. From the 16 dysregulated genes above this threshold, six genes with the highest changes in gene expression were chosen for validation via qPCR (*Apoe, Dhcr24, Pcsk9, Soat2, Cyp11al,* and *Abca1*).

Changes in gene expression after nerve injury and recovery

To understand the recovery process in nerve injured control animals given saline, I compared gene expression post injection (day 14) and 4 weeks later. The greatest changes were found in the upregulation of *Pcsk9* and *Soat2*. *Pcsk9* encodes a protein involved in controlling LDL cholesterol levels by regulating the LDL receptor and has been implicated in neuronal apoptosis, inflammation and microglial/astrocytic hyperactivation (Apaijai et al., 2019; Zhao et al., 2017). SOAT2 is responsible for converting cholesterol and fatty acids to cholesteryl esters (Wang et al., 2017). However, when lipid levels are low, SOAT2 is ubiquitylated for degradation (Wang et al., 2017). Together, the increase in gene expression over the span of recovery could indicate that cholesterol, LDL cholesterol and fatty acids are higher in nerve injured animals 4 weeks after injection. As these are constituents of the myelin membrane, it could be suggested that this recovery timepoint in CCI animals involves remyelination. In models of CCI where the sciatic nerve is released one week after constriction, there is remyelination of the sciatic nerve 5 weeks after injury (Chen et al., 2021). Although this is a modified model and is focused on the spinal cord, it supports the idea

that remyelination may be taking place during recovery in the CCI model 4 weeks post injection (6 weeks post injury).

Morphine induces temporal changes in gene expression following nerve injury

When comparing morphine treatment of nerve injury over the span of recovery, there are two dysregulated genes that are of particular interest. *Abca1* encodes the primary transporter for cholesterol efflux which is responsible for apoE secretion (Saher & Stumpf, 2015). Depletion of *Abca1* has been shown experimentally to decrease myelination and oligodendrogenesis (L. Li et al., 2020). At the week 4 timepoint in morphine treatment of nerve injury, this could suggest that myelin and oligodendroglia are negatively impacted, which may contribute to the delay in recovery. In contrast, *Cyp11a1*, the mitochondrial cytochrome p450 enzyme that converts cholesterol to pregnenolone, is upregulated. Oligodendrocytes produce high levels of pregnenolone via CYP11A1 (Zwain & Yen, 1999) and the highest levels of Cyp11a1 in the CNS were found in the spinal cord. Beyond serving as a precursor to all other neurosteroids, pregnenolone may increase myelination in the periphery through promoting Schwann cell differentiation to the myelinating phenotype (Zhu & Glaser, 2008). It should be noted that a more recent study has suggested that pregnenolone is synthesized in human glia via a different mitochondrial cytochrome p450 enzyme than Cyp11a1 (Y. C. Lin et al., 2022). Given the conflicting literature, more experiments are needed to determine the presence and role of Cyp11a1 within glia. *Cyp11a1* upregulation may be an attempt to myelinate at the week 4 timepoint after morphine treatment of nerve injury.

Candidates for morphine versus saline treatment of nerve injury at week 4

In the comparison between morphine and saline treated animals at 4 weeks post injection, the most dysregulated candidates are *Apoe* and *Dhcr24*. *Apoe* encodes apolipoprotein E, the main protein responsible for the transport of cholesterol and is mainly expressed in microglia and astrocytes (Meschkat et al., 2022). In adulthood, astrocytes supply oligodendrocytes with cholesterol via ApoE for the maintenance of myelin (Saher & Stumpf, 2015). After nerve injury, ApoE may aid in the regeneration of myelin through coordinating cholesterol transport (Ignatius et al., 1987). This could suggest that in morphine treated CCI animals, a downregulation of *Apoe* could be delaying remyelination and prolonging recovery. Furthermore, previous studies have linked ApoE with chronic pain states in humans and rodents (Tansley et al., 2022). In a rodent model of chronic pain, the upregulation of *Apoe* in was specifically identified in microglia, which suggests that in this state, ApoE cholesterol efflux is required by microglia after their phagocytosis of myelin debris (Tansley et al., 2022). As *Apoe* is downregulated in the morphine treated CCI animals at the week 4 time point, this could indicate that proper inflammatory responses by microglia are not occurring to clear myelin debris, which could be prolonging the neuropathic pain state and delaying remyelination.

Another gene of interest, *Dhcr24,* encodes the enzyme that catalyzes the terminal step in cholesterol synthesis; the conversion of desmosterol to cholesterol (Zerenturk et al., 2013). In response to demyelination, *Dhcr24* is downregulated in microglia and astrocytes, which increases desmosterol levels, in turn activating the liver X receptor (LXR) (Berghoff et al., 2021). This signaling decreases inflammation and it is a key cellular step in oligodendrocyte differentiation and remyelination (Berghoff et al., 2021). In morphine treated animals, the downregulation of *Dhcr24* is possibly in response to demyelination.

From the six genes chosen for validation, *Cyp11a1* was significantly increased in nerve injured animals given morphine compared to those given saline at the recovery timepoint (week 4) and when compared to nerve injured animals given morphine at the earlier timepoint post injection (day 14). The upregulation of *Cyp11a1* in nerve injured animals given morphine versus saline at the week 4 timepoint is a result unique to the validation qPCRs, as this was not observed in the profiling arrays. Although these preliminary data suggest there may be a significant difference in *Cyp11a1*, this requires further investigation and confirmation at the protein level. In the literature, the inhibition of *Cyp11a1* has been shown to decrease the development of mechanical allodynia in a neuropathic pain model, however, the impacts of morphine administration on nerve injury were not studied (Choi et al., 2019).

Taken together, the profiles of up and down regulation in these six candidate genes could indicate that morphine treatment is delaying recovery from nerve injury by disrupting lipid metabolism and signaling important for myelination. As glia are responsible for the production, distribution and integration of the lipids required for myelination, delays in this process could imply that astrocytes, oligodendrocytes, and microglia are impacted by morphine treatment of nerve injury. Astrocytes are responsible for cholesterol production and transport through lipoproteins to support myelination in adulthood, therefore changes in cholesterol metabolism and lipoprotein signaling may involve this cell type (Camargo et al., 2017). As oligodendrocytes are responsible for myelin maintenance and the production of fatty acids, perturbations of this process could imply that oligodendrocytes are impacted by morphine treatment of nerve injury (Dimas et al., 2019). Alterations in microglial lipid metabolism have been implicated in pain (Navia-Pelaez et al., 2021) and recently, microglia were shown to regulate myelin integrity through their interactions with oligodendrocytes (McNamara et al., 2023). Given the relationship between lipids and glia, further

experiments are needed to elucidate which glia are implicated in the lipid metabolism changes after morphine treatment of nerve injury.

Limitations and Future Directions

My findings indicate that morphine treatment ten days after nerve injury prolongs recovery in mice. Furthermore, I found that genes related to cholesterol metabolic processes and plasma lipoprotein signaling are differentially expressed in response to morphine treatment of neuropathic pain. However, there are limitations to the approaches used and considerations when interpretating these data.

First, all experiments were conducted in one sex (male) to limit the number of animals required, as these experiments required multiple large groups. As sex differences are important to consider, especially when studying pain, these experiments will be repeated in female mice. Sex differences are primarily due to differences in sex hormones. Since lipids are the precursors to sex hormones, I anticipate that morphine treatment of CCI female mice will result in a different profile of lipid related changes. I also hypothesize that female mice will have an even longer prolonging of neuropathic pain due to morphine than male mice due to sex steroid hormone differences and the prevalence of chronic pain in female humans (Sorge & Totsch, 2017).

Further, all RNA work was done using lumbar spinal cord homogenate. This limits the findings because it does not allow conclusions to be made regarding the localization of gene expression changes within the spinal cord (dorsal, ventral, ipsilateral, contralateral, white matter, grey matter) or a particular cell type (neurons, astrocytes, microglia, oligodendrocytes). However, the purpose of these experiments was to screen broadly to identify new candidates and by not selecting a certain region or cell type, this approach was unbiased and potentially allows for a more global

understanding of the changes occurring within the lumbar spinal cord. Future experiments would focus on elucidating which cell types are involved, specifically oligodendrocytes as they are understudied in the context of pain and opioid administration. It would be important to understand the role of different cells within the context of morphine prolonged neuropathic pain when developing treatments that could be co-administered with morphine to potentially mitigate the negative side effects. Another cell type worth considering are Schwann cells, as they are the myelinating cells of the PNS. In future experiments, I would assess whether there are converging mechanisms centrally and peripherally by isolating the sciatic nerves from morphine treated CCI animals. A common candidate between the central and peripheral nervous systems could potentially increase the efficacy of a candidate targeting treatment, especially since morphine is administered systemically and could be acting at multiple sites of pain signaling.

For feasibility of the experiments, only two timepoints were chosen (4 weeks post injection and 14 days post injury). The week 4 timepoint was chosen based on behavioral differences between the morphine and saline treated CCI animals, and gene expression changes could be altered earlier than changes are seen behaviorally. Examining other timepoints along the recovery timeline could help in identifying additional candidates. In addition, only 6 out of 16 dysregulated genes from the profiling array results were chosen for validation. In further studies, it would be important to perform validation experiments on the remaining 10 genes to assess whether they are confirmed via qPCR. In order to test causality of the validated candidate *Cyp11a1*, I would perform validation experiments in vivo to see whether blocking Cyp11a1 four weeks after nerve injured animals are given morphine would accelerate their recovery.

To elucidate changes in lipids, gene expression changes in enzymes and proteins crucial for cholesterol metabolic processes and lipoprotein signaling were examined. This was chosen as an

initial screening step; however, future directions should look at lipids more directly. As lipids are the major component of myelin membranes, studying changes in myelin would be a future avenue of investigation. Exploring changes in myelin can be challenging, as typical techniques can disrupt spatial information and lack specificity (Teo et al., 2021). Nevertheless, future directions could include employing a new highly sensitive and quantitative technique, Nile Red fluorescence spectroscopy, to identify physicochemical changes in spinal myelin after morphine treatment of CCI (Teo et al., 2021). The dysregulation in lipid-related genes highlights exciting new avenues for study, especially as they impact glia and the crucial processes they support, such as myelination.

Significance

My project examined the consequence of morphine treatment on neuropathic pain, indicating that morphine treatment after neuropathic pain has been established delays pain recovery. The goal of this work is not to add to the negative bias surrounding opioids – they are powerful analgesics with a time and place in society. By studying the cellular impact of opioids in the different contexts they are used in, we can better understand how to harness their benefits. For the first time, lipid dysregulation was screened to determine impact of opioid treatment of neuropathic pain within the spinal cord. I explored whether morphine may prolong neuropathic pain through its actions on cholesterol metabolism and lipoprotein signaling pathways within the spinal cord. The newly identified lipid-related candidate genes could expand the pain field's knowledge of how opioids and neuropathy intersect. Understanding the changes in lipids and developing ways to target them could help mitigate adverse opioid effects and improve the treatment options for people suffering from neuropathic pain.

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