CHARACTERIZATION OF SPINAL CORD INJURY AND SPINAL CORD INJURY INDUCED NEUROPATHIC PAIN

by

AMANDA MOHAIMANY-APONTE

CANDACE L. FLOYD, COMMITTEE CHAIR
KENESHIA M. KIRKSEY
SEAN D. MCALLISTER
TIMOTHY J. NESS
ROBERT E. SORGE

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2018

Copyright by

Amanda Mohaimany-Aponte

2018

CHARACTERIZATION OF SPINAL CORD INJURY AND SPINAL CORD INJURY INDUCED NEUROPATHIC PAIN

AMANDA MOHAIMANY-APONTE

PATHOBIOLOGY AND MOLECULAR MEDICINE

ABSTRACT

Spinal cord injury (SCI) affects between 40 and 80 million people globally. Within the United States over 400,000 individuals live with SCI, and annually over 17,000 individuals are added to this population. SCI patients are afflicted with a myriad of issues, one of which being SCI induced neuropathic pain (SCI-NP). Up to 80% of SCI patients go on to develop SCI-NP, which has been shown to last chronically and present itself as evoked pain, spontaneous pain, or a combination of both. SCI-NP is a top concern for SCI patients, often listed before functional recovery. SCI-NP has been shown to greatly diminish quality of life, as there are poor therapeutic options for this issue. Several issues complicating the development of therapeutics is that the root cause of SCI is accidental trauma and not an underlying disease thus introducing high variability between patients; additionally, level and severity of injury are not correlated to SCI-NP development. These issues cause complications determining which patients are at high risk for developing this pain disorder as well as difficulty in developing treatments for SCI-NP. With these issues in mind, the goals of the studies conducted were two-fold. First, to better understand contributors to SCI-NP development, and secondly evaluating a therapeutic for SCI-NP. The first goal was investigated by exposing two strains of rodents with differing levels of stress responsiveness to a chronic stress paradigm prior to SCI. The second goal was investigated by administering cannabidiol (CBD) acutely after injury. Both studies utilized an incomplete cervical injury model and behavioral assessments testing motor and senso-

ry function. In the stress study, it was found that pre-injury stress increased incidence of response to cold stimuli in rodents hyper-responsive to stress, indicating that chronic stress prior to injury exacerbates SCI-NP. In the CBD study, it was determined that CBD administration acutely after injury imparted protective effects against SCI-NP in both sexes, with trends of greater protection in females; indicating that CBD is a viable therapeutic for SCI-NP. Collectively, the body of work presented aimed to provide understanding into SCI-NP and provide a platform to provide therapies for this disorder.

Keywords: spinal cord injury, neuropathic pain, cold allodynia, chronic mild stress, cannabidiol, sex differences

DEDICATION

Dedicated to Steve, thank you for brightening up the room and providing me with a breath of fresh air.

ACKNOWLEDGMENTS

Many people have aided me throughout my graduate career, far too numerous to list, however there are individuals who have been instrumental in my journey. First, I would like to thank my mentor Dr. Candace Floyd who has guided, taught, and supported me. You allowed me the independence to pursue not only my own scientific quests, but non-academic interests which have greatly shaped my future. This brings up the Floyd Lab, which other graduate students and faculty have come to regard as a hard-working, independent, tight-knit group – to which they are not wrong. To my lab mates, past and present, I want to thank you for your support and guidance on countless presentations, data, and life lessons and events. Halloweens will never be the same. Betty and Tracy, thank you. Your help and advice has been crucial to my success. Jess, my definitely-not-co-dependent lab mate, thank you for the laughs, conversation, late night science sessions, competitive spirit, and being a sounding board for my thoughts. I value what you have given me and hope I have given you as much. Art, thank you for writing with me all those nights and keeping me motivated. Team R.A.M. J.A.M. and Lady Legasus, thank you for the camaraderie, fun, and helping me accomplish life goals - I never thought I would ever run a half marathon, let alone three. My friends, thank you for your support, acceptance, and love.

To my collaborators, Drs. McAllister, Ness, and Robbins, thank you for all your help on my projects. You all have helped tremendously, thank you for your time and generosity.

To my committee, your expertise, advice, and direction have aided me in the evolution of these projects and helped me push the field further, thank you all.

To my parents, thank you for all the sacrifices you have made to provide me with every opportunity to excel. Thank you for coming to this country with nothing and building a life full of love and encouragement. I hope I have made you proud, and have proven I am the best, albeit only, daughter you have. Dad, with all the love in my heart, I hope this document helps you in finally understanding what I actually did at UAB. Allow me to answer your three requisite questions before you begin. No, I am not in any more classes. Yes, I am still in lab. Yes, it is the same lab. Mom, thank you for your support and love, and understanding more about what I do than dad. To my older brother, Peyvand. Thank you for using your vacation time to come visit me and do brotherly things for me like change my wiper blades. The little things you have done for me amount to a lot of love. To my future sister-in-law Cortni, thank you for coming to visit and being a part of our family, especially helping our mom with the internet. To my little brother, Ryan. Thank you for being my roommate and letting me eat your left overs on days when I would come home too tired to be an adult and make food, and for loving me enough to put up with me. To our lil dergs Bella and Shell, your unconditional love and loyalty have been amazing. Lastly, I want to thank all my haters, for the greatest revenge is success.

TABLE OF CONTENTS

Page

ABSTRACT iii

DEDICATION v

ACKNOWLEDGMENTS vi

LIST OF TABLES x

LIST OF FIGURES xi

LIST OF ABBREVIATIONS xiii

INTRODUCTION 1

Clinical Perspective of Spinal Cord Injury 1
Epidemiology of Spinal Cord Injury 1
Sex Differences in SCI 3
Epidemiology and Current Treatment of SCI Induced Neuropathic Pain 4
Pathophysiology of SCI-NP 5
Sex Effects on 2^{O} Injury 9
Animal Models of SCI and SCI-NP 11

Stress States, Physiological Effects, and the Effects of Stress on Pain 14
Acute and Chronic Stress 14
Models of Stress: Modes of Stress, Animals, and Strain Differences 16
Effects of Chronic Stress on SCI and Pain Disorders 18

Cannabidiol: Therapeutic Potential for SCI-NP 19
Cannabidiol: Clinical and Experimental Treatment for NP 19

Significance 20

CHARACTERIZATION OF CHRONIC, MILD STRESS PRIOR TO SPINAL CORD INJURY IN MALE SPRAGUE DAWLEY AND LEWIS RATS 21

POTENTIAL THERAPEUTIC EFFICACY OF CANNABIDIOL ON SECONDARY INJURY POST SPINAL CORD INJURY 58

CANNABIDIOL ADMINISTRATION AFTER SPINAL CORD INJURY REDUCES ALLODYNIA IN BOTH MALE AND FEMALE RATS, WITH MOST ROBUST EFFECTS IN FEMALES 84

DISCUSSION 122

Overarching Goals 122
Major Findings 122
SCI Model 122
CBD… 126
Sex Differences 127
Chronic Stress 130
Conclusion 132
Future Directions 133

LIST OF REFERENCES 137

APPENDICES

A IACUC APPROVAL FORMS 151

LIST OF TABLES

Tables *Page*

INTRODUCTION

1 Models of traumatic spinal cord injury 12

LIST OF FIGURES

Figure *Page*

INTRODUCTION

1 Epidemiology of spinal cord injury 2

2 Spinal cord injury and secondary injury 7

3 Activation and deactivation of the hypothalamic pituitary adrenal axis................15

CHARACTERIZATION OF CHRONIC, MILD STRESS PRIOR TO SPINAL CORD INJURY IN MALE SPRAGUE DAWLEY AND LEWIS RATS

1 Spinal cord injury significantly decreases neuronal numbers and white matter at the epicenter of injury and is not affected by stress 45

2 Injury and rodent strain, but not stress exposure, affect motor function 46

3 Stress differences between strains and the effects of stress exposure 48

4 Body mass gains are affected by injury and strain 49

5 Thermal hyperalgesia is not affected by injury or stress exposure, with intermittent effects of strain 50

6 Mechanical sensitivity is not affected by strain, stress exposure, or injury...........51

7 Injury increases cold sensitivity and is affected by strain and stress exposure 52

POTENTIAL THERAPEUTIC EFFICACY OF CANNABIDIOL ON SECONDARY INJURY POST SPINAL CORD INJURY

1 Secondary injury spreads damage..73

CANNABIDIOL ADMINISTRATION AFTER SPINAL CORD INJURY REDUCES ALLODYNIA IN BOTH MALE AND FEMALE RATS, WITH MOST ROBUST EFFECTS IN FEMALES

1 Cannabidiol plasma concentrations 107

2 Body mass gains are affected by injury and drug treatment in males 108

3 Motor function is affected by injury with intermittent effects of drug treatment and not affected by sex 109

4 Mechanical sensitivity is not affected by injury, drug treatment, or sex 110

5 Cold sensitivity if affected by injury and drug treatment in both sexes 111

6 Facial grimace scores are affected by injury and application of cold stimuli in both sexes..112

7 Injury induces autophagic behavior in both sexes and is affected by drug treatment 113

8 Injury diminishes neuronal numbers with no effect of sex or drug treatment 114

9 White matter area is negatively impacts by injury, with sex differences and drug treatment effects observed 115

LIST OF ABBREVIATIONS

1^{O} injury	Primary injury
2^{O} injury	Secondary injury
3-NT	1,3-nitrotyrosine
5-HT	5-hydroxytryptamine
5-HT_{1A}	5-HT subtype 1A
ACTH	Adrenocorticotropin releasing hormone
AMPA	2-amino-3-(4-butyl-3-hydroxylsoxazol-5-yl) propionic acid
ANOVA	Analysis of variance
AU	Action units
BL	Baseline
C	Cervical
C. sativa	*Cannabis sativa*
C1	Cervical region vertebral level 1
C5	Cervical region vertebral level 5
Calpain	Calcium-activated neutral proteinase
CB	Cannbinoid
CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
CBD	Cannabidiol
cm	Centimeter
CNS	Central nervous system
CORT	Cortisol
CRH	Corticotropin releasing hormone
d	Tabular value based on pattern of paw responses
E	Estrogen
ERK	Extracellular signal related kinases
F	Female
F 344	Fischer 344
FKBP 51	FK506 binding protein 51
FS	Foot shock
g	Gram
GABA	Gamma-Aminobutyric acid
GC	Glucocorticoid
GPCR	G protein-coupled receptor
HPA	Hypothalamic pituitary adrenal
HPLC	High-performance liquid chromatographer
i.p.	Intraperitoneal

I.V.	Intravenous
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IRR	Inter-rate reliability
k	Constant of the average difference of force between filaments
kdyne	kilodyne
kg	Kiligram
L	Lumbar
LAM	Laminectomy
LEW	Lewis
M	Molar
M	Male
m/z	Mass and charge
mA	Milliamp
mg	Milligram
mL	Milliliter
mm	Millimeter
NF-κB	Nuclear factor-kappa b
ng	Nanogram
NMDA	N-methyl-D-Aspartate
NMDA	N-methyl-D-aspartate
NP	Neuropathic pain
p.o.	Post operation
PBS	Phosphate buffered saline
PNS	Peripheral nervous system
PPAR	Peroxisome proliferator-activated receptors
PPARα	Peroxisome proliferator-activated receptor alpha
PPARβ	Peroxisome proliferator-activated receptor beta
PPARγ	Peroxisome proliferator-activated receptor gamma
PTSD	Post-traumatic stress disorder
ROS	Reactive oxygen species
rpm	Revolution per minute
S	Sacral
S	Stress
s	Second
SCI	Spinal cord injury
SCI-NP	Spinal cord injury induced neuropathic pain
SD	Sprague Dawley
s.e.m.	Standard error of the mean
SMA	Superior mesenteric artery
T	Thoracic
T1	Thoracic region vertebral level 1
T2	Thoracic region vertebral level 2

THC	Delta9-tetrahydrocannabinol
TNF-α	Tumor necrosis factor alpha
TRP	Transient receptor potential
TRPA1	Transient receptor potential ankyrin 1
TRPV1	transient receptor potential vanilloid receptor
uL	Microliter
VEH	Vehicle
Xf	Final filament force
Δ9-THC	Delta9-tetrahydrocannabinol

INTRODUCTION

Clinical Perspective of Spinal Cord Injury

Epidemiology of Spinal Cord Injury

Globally, spinal cord injury (SCI) is estimated to affect between 40 to 80 million individuals [1]. Within the United States of America, over 400,000 individuals live with chronic SCI with approximately 17,000 cases each year [2]. The primary cause of injury is largely due to accidental trauma, with the leading cause being vehicular accidents comprising 38% followed by falls making up 30.5% of SCI cases. The nature of the injury depends on the location and type of injury.

Type of injury can be broken into two categories, complete and incomplete. Complete SCI results in complete loss of motor and sensory function below the site of injury, whereas incomplete SCI results in partial loss of motor and sensory function below the area of injury. The area affected by injury is dictated by the level of SCI, resulting in either paraplegia or tetraplegia (figure 1). The spinal cord can be divided into four sections. The most rostral section being cervical (C), followed by thoracic (T), lumbar (L), and sacral (S). Paraplegia is classified as the lower extremities affected by SCI, indicating that the injury has occurred at or below the second vertebral level of the T region (T2). Tetraplegia, where all four extremities are affected by SCI, occurs when an injury takes place between C1 and T1 [2, 3]. The C region of the spinal cord is the most frequently injured region due to the neck being an area with relatively low protection,

rotational ability, and weight of the head [2, 4-6]. Tetraplegia represents the most common type of SCI accounting for 58% of injuries with incomplete tetraplegia at a frequency of 45% of SCI cases [1, 2].

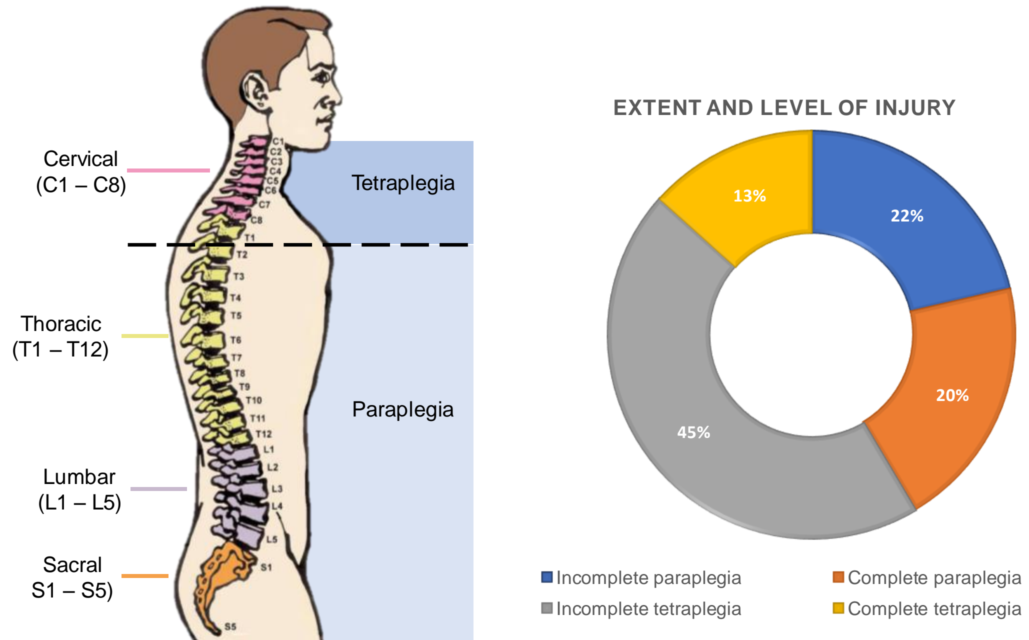


Figure 1: Epidemiology of spinal cord injury. Location and severity of SCI dictate type of and extent of injury. SCI occurring between C1 and T1 result in tetraplegia, where all four limbs are affected; and lesions occurring at T2 and below result in paraplegia where only the lower extremities are affected. Injuries can be complete or incomplete, complete loss of motor and sensory function or incomplete loss below the lesion area. The most common type of SCI is incomplete tetraplegia, followed by incomplete paraplegia, complete paraplegia, and incomplete tetraplegia.

Individuals who sustain an SCI go on to develop a myriad of issues, some beginning acutely post injury within hours to days and others manifesting more chronically, weeks to months post injury [7-10]. Injury to the spinal cord results in a range of issues due to resultant injury to the central nervous system (CNS). The CNS is comprised of the brain and spinal cord, and is involved in integrating internal and external information, coordinating central and peripheral responses to stimuli, and regulating central and

peripheral functions with the spinal cord serving as relay between the brain and peripheral systems. Damage occurring to this relay system causes issues with both central and peripheral function [3]. Acutely post injury, SCI patients exhibit motor and sensory loss at and below the site of injury[11]. Beyond these two hallmarks of injury, patients go on to develop autonomic dysreflexia, pressure sores, metabolic disorders, pain disorders, as well as a multitude of other issues [7, 8, 12-19].

Sex Differences in SCI

Within the SCI population, there is a vast male skew, with a 4:1 male to female ratio [2]. Both physical and biological factors contribute to this sex ratio difference. Males, for instance, have been shown to make a greater proportion of individuals injured in motor vehicle accidents, with a rate of injury approximately three to four times greater than women [20]. Considering that motor accidents comprise 38% of SCI cases, the greater rate of injury in males would contribute to the sex skew [2].

Biologically, estrogen (E) and E related compounds such as estradiol have been shown to exert protective effects post-SCI. In an animal model of SCI, it has been observed that females have decreased area of injury, increased myelin preservation, increased neuronal survival, and improved motor function [21-24]. Studies investigating the effects of E administration post-SCI in animal models have shown that exogenous E administration in males extends the same protective effects observed in females [25-32].

Though the incidence of injury is lower in females and E exerts protective effects post-SCI, females that sustain SCI have clinical outcomes similar to males. Females, like males, exhibit motor and sensory deficits that occur acutely post injury and persist

chronically with little recovery, as well as autonomic dysregulation, metabolic disorders, and pain development which highlights the importance of investigating both males and females in regards to SCI [8, 12-15].

SCI Induced Neuropathic Pain: Clinical Significance, Characterization, and Investigation

Epidemiology and Current Treatment of SCI Induced Neuropathic Pain

Due to the resultant injury to the spinal cord, damaged nerves abnormally signal which can cause neuropathic pain (SCI-NP). Between 60% and 80% of SCI patients go on to develop SCI-NP [7, 8, 16]. This pain disorder is frequently listed as a top priority for patients, as it has been shown to greatly contribute to diminished quality of life. Patients that go on to develop SCI-NP can experience intermittent or constant episodes which present as allodynia, pain response to non-painful stimuli, and/or hyperalgesia, heightened pain response to painful stimuli. SCI-NP develops around 1 month post-injury and is experienced below the lesion level, but development is not correlated with level of injury. Due to the accidental nature of SCI, predictability of SCI-NP development is low and highly variability between patients. In totality, this creates great difficulty in SCI-NP management and therapeutic options [7, 8, 33, 34].

Therapeutic options for SCI-NP mainly rely on decreasing neuronal excitability, as signaling from damaged nerves is a major underlying cause of this pain disorder. Current drugs utilized to treat SCI-NP aim to inhibit or increase inhibition of nerve signaling and fall into the categories of opioids, anti-epileptics, and anti-depressants. However, the estimated rate of efficacy for this pain intervention is only estimated to be 50%, which

leaves many patients still living with the consequences of SCI-NP [35]. Patients with SCI-NP report decreased quality of life, which manifests itself as increased rates of depression, anxiety, sleep loss, and inability to work [7-9, 33, 34]. Thus, the negative impacts on patient quality of life coupled the lack of effective therapeutics demonstrates a significant and unmet need to further evaluate this topic.

Pathophysiology of SCI-NP

Considering the accidental nature of SCI, the SCI patient population is widely diverse in regards to medical history as underlying medical disorders are a small facet of the SCI population [1, 2]. Therefore, when examining SCI-NP it is important to focus on what is common between patients, as this will improve treatment regimens by providing insight on areas to target.

As discussed earlier, abnormal nerve signaling is a major contributor to SCI-NP [36-39]. However, considering that nerve damage is unavoidable in the context of SCI, it is important to examine aspects that contribute to SCI-NP development post injury. A hallmark of SCI and other nerve injuries resulting in pain is central sensitization [37]. Central sensitization can develop when changes to the CNS occur and in the case of SCI, the change is induced by damage. The resultant damage causes the CNS to change in the way it responds to peripheral stimuli and neuronal inputs, as well as changes neurons within the CNS [40].

Studies characterizing central sensitization have found that nerves in the dorsal horn, the region where peripheral sensory inputs synapse with the CNS, develop spontaneous and heightened activity, exhibit a decrease in threshold to response, increase their receptive

field, and react to a variety of stimuli. Ultimately, these changes result in pain no longer being confined to area of injury alone and extending below the area of injury, as is often the case in SCI. In addition to increased receptive field, pain sensations are no longer fixed to stimuli, which can lead to development of allodynia. In addition to allodynia, this uncoupling leads to pain responses which are not dependent on stimuli duration, intensity, or presence. Hence, potential development of spontaneous pain and hyperalgesic pain. In clinical cases, patients can experience one of these pain phenotypes or a combination of these pain phenotypes.[3, 7, 8, 40, 41].

Considering the role of central sensitization in SCI-NP development, the root cause of central sensitization must also be investigated. As mentioned earlier, the cause of central sensitization following SCI is the damage induced by the injury. There are two main injury phases that occur in SCI – primary (1°) injury and secondary (2°) injury. The first stage, 1° injury, is the mechanical to the cord itself and is characterized by shearing, tearing, and compression forces that injure not only neurons, but damage the vasculature, laminae, and myelin [42-44].

Damage created by the 1° injury, sets off a myriad of events which induces and feeds the subsequent stages of 2° injury which begins within minutes. Characterized as a cascade effect, 2° injury can be broken into sub-stages of acute, sub-acute, and chronic 2° injury. The acute stage of 2° injury begins within minutes of the initial trauma and lasts for several days. Vascular disruption to the cord induces changes in blood flow such as edema and ischemia; depolarization events cause ionic dysregulation; and immediate cell death induces inflammation and the recruitment and activation of immune cells. The sub-acute stage, which begins several days following the initial mechanical injury and can last

weeks after injury, carries similar characteristics of the acute stage, such as edema, ionic dysregulation, inflammation, and immune cell recruitment and activation (Figure 2) [11, 43, 45, 46].

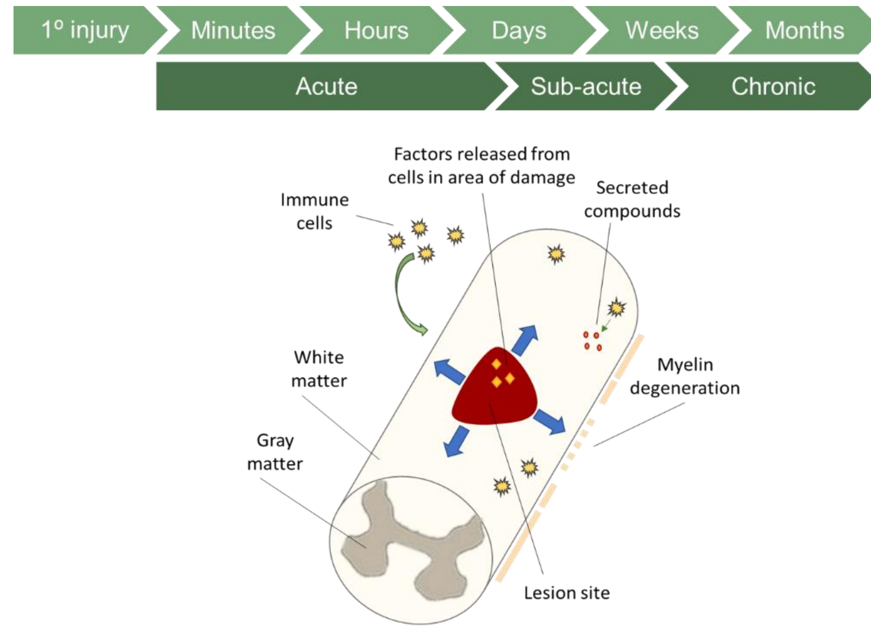


Figure 2: Spinal cord injury and secondary injury. SCI induces a 2° injury cascade which results in the spread of damage outward, rostral and caudal, from the lesion site thus increasing the area of damage. In the acute stages of 2° injury, injured and dying cells release inflammatory compounds and ROS, as well as compounds that contribute to excitotoxicity, namely glutamate. In the sub-acute stages of 2° injury, immune cells are activated and recruited to the site of injury where they infiltrate into the tissue and contribute to inflammation via secretion of inflammatory compounds. In the chronic stages of 2° injury, apoptosis of damaged neuronal cells occurs in addition to myelin degeneration.

The effects of these can further cascade into glutamatergic excitotoxicity, production of free radicals, and immune cell infiltration. Excitotoxicity leads to neuronal damage and death through influxes of calcium into the cell mediated by glutamate binding to receptors that open ion channels. Free radicals, which are generated during normal metabolic processes as well as during phagocytosis, can cause cellular damage when the

ratio between anti-oxidant species and reactive oxygen species (ROS) are out of sync. In the context of SCI, the damage caused by the initial injury induces activation of ROS generating enzymes. Due to the increased amount of ROS present, the anti-oxidants available to neutralize these compounds are decreased quite rapidly. This imbalance allows for ROS to cause damage such as lipid peroxidation. As mentioned, ROS can be generated by phagocytotic cells, such as macrophages. Resident macrophages, microglia, are activated acutely after injury, with circulating leukocytes being recruited to and infiltrating to the site of injury in the beginning of the subacute phase of 2° injury [47-52].

During the early phases of 2° injury, macrophages and microglia take on a pro-inflammatory phenotype, peaking at around seven days post-SCI [37]. Also, reaching peak numbers at approximately one week are T cells and neutrophils, both of which have a pro-inflammatory expression profile at this stage of injury [53]. Approximately two months after SCI, macrophages and microglia begin to diminish in number and reach roughly half of their total population [54]. The resulting lipid peroxidation, increased inflammation, apoptotic events, and necrosis of neuronal cells, leads to increased damage rostrally and caudally outward from the initial site of injury. The chronic stage of 2° injury begins approximately one month after SCI with hallmarks including demyelination, apoptotic activity, cavitation, glial scar formation, neural regeneration and sprouting, and phenotypic changes in neurons.

The resultant effects of demyelination on the CNS is poor conductance of action potentials, which contributes to motor and sensory dysfunction observed post injury [55, 56]. Glial scarring, which begins several weeks after injury, is characterized by glial

cells, primarily astrocytes and microglia walling off the injury site. Within the scar, astrocytes produce compounds that inhibit penetration such as proteoglycans and glycoproteins which ultimately disallow the regenerating neurons to pass through the lesion area [57, 58]. In addition to neural regeneration, surviving neurons begin to undergo phenotypic changes. The definitive characteristics are increased spontaneous activity, enlarged receptor fields, and decreased depolarization thresholds. The mechanisms for these phenotypic changes are the results of changes in membrane potential, the switch of nociceptive neurons to wide-dynamic responding neurons, and increased expression of excitatory receptors such as N-methyl-D-Aspartate (NMDA) [37, 40]. The chronic stage of 2° injury has been shown to persist for months to a year after SCI, which like the sub-acute stage, expands damage outward from the initial injury site [11, 37, 42-44, 46].

The increased damage caused by the 2° injury cascade lays the foundation for SCI-NP development. In animal models of nerve injury and SCI, studies have demonstrated that therapeutically targeting aspects of 2° injury results in a reduction in NP development [59-70]. Considering that 2° injury is common to all SCI patients and that diminishing aspects of 2° injury in animal models of SCI and nerve injury decreases NP, better understanding of the mechanisms behind 2° injury will aid in in the development of treatments for SCI-NP.

Sex Effects on 2° Injury

As discussed earlier, sex differences exist in the context of SCI. At chronic endpoints, sex differences confer greater protection in females compared to males in the form of greater

white matter preservation, neuroprotection, and improved motor function [21-24]. Studies seeking to elucidate underlying reasons for these differences have found that differences exist within the 2° injury cascade. For example, the concentration of glutamate in the CNS is affected by the estrous stage of a female rodent. Specifically, glutamate concentrations were found to be the lowest in the diestrous phase of the estrous cycle when E levels are low [71]. Considering the role of glutamate in excitotoxicity, there could be sex differences between males and females in this aspect. Immune cell activation and infiltration is present in both sexes post SCI, as previously discussed, but it has been shown that E affects both T cells infiltration and microglia activation. A study investigating sex differences in regards to the role of immune cells and NP found that E increased T cell presence and decreased the migration of macrophages [72].

In totality, these sex differences can have effects on outcomes post-SCI, specifically SCI-NP. As discussed earlier, the root cause of SCI-NP is abnormal signaling from damaged nerves. These damages nerves have been shown to have increased excitability, increased spontaneous signaling, and changes in receptor and channel expression chronically post injury [36-40]. Considering the differences observed in 2° injury, there could be differences in contributors to SCI-NP development between males and females. The aforementioned study examining sex differences in regards to the role of immune cells and NP found that macrophages contribute to SCI-NP more greatly in males compared to females, and that T cells contribute to NP more greatly in females compared to males [72]. Though both sexes developed pain, the factors that contributed to pain differed, highlighting the need to investigate sex differences in SCI and SCI-NP as well as factors that contribute to SCI-NP development.

Animal Models of SCI and SCI-NP

Animal models of traumatic SCI fall into three main categories – compression, transection, and contusion. Each model has their advantages and selection of said model is dependent on what aspect of SCI is being investigated (table 1). Compression models involve physical compression of the spinal cord via use of forceps, balloons, clips, or application of weights. Advantages of the compression model are the ability to investigate ischemia and reperfusion and effect of compression duration and intensity. In addition to these advantages, the compression model is relatively simple and reflects compression forces observed clinically as bone fragments, tissue swelling, and blood flow disruptions can cause compression and pressure forces [45, 73]. Disadvantages of the compression model are difficulty in measuring the compression device interaction with the tissue – velocity of interaction, actual force exerted, and extent of damage. Transection models of SCI are relatively simple to perform and provide insight into regeneration post SCI, making this model advantageous in investigating tissue engineering. However, this model is a poor reflection of clinical SCI cases as transection is rare, making roughly 1% of SCI cases [74]. Contusion models of SCI can be performed using weight drop, impactors, or air gun. Advantages of this model include wide use within the SCI field among a range of animals such as mice, rat, dogs, and pigs, validation of use, ability to monitor and record contusion impact force and duration, and clinical relevance, as the vast majority of SCI cases are caused by transient contusion forces [75]. Considering these factors, the contusion model allows the ability to investigate many aspects of SCI as it relates to the clinical population. Disadvantages of

this model are the ability to control weight bounce in weight drop models and duration of impact in non-impactor devices [76-78].

Critical to SCI models are area and type of injury. As discussed earlier, area of injury can be divided into four regions, C, T, L, and S, and type of injury can be divided into two categories, complete and incomplete [2]. A study reviewing animal models of SCI determined that T level injuries are the most common area of injury at 81%, followed by C at 12%, L at 5%, and S at 0.7% of 2,209 literature articles. The study further determined that contusion, transection, and compression injuries were found to make up 41%, 32.5%, and 19.4% of the literature articles surveyed, respectively [77].

Table 1: Models of traumatic spinal cord injury

Injury Model	Advantages	Disadvantages	Literature Relevance	Clinical Relevance
Compression	Duration and intensity of compression forces	Replication and reproducibility of forces exerted on tissue	41% of SCI models	Compression forces common in SCI cases
	Vascular changes and disruptions			
	Used in a wide variety of animal models			Vascular changes and disruptions common in SCI cases
	Simple and inexpensive method			
Transection	Neural regeneration	Inconsistent reproducibility	32.5% of SCI models	Low clinical incidence
	Tissue and scaffold engineering			
	Used in a wide variety of animal models	Poor incomplete injury consistentcy		
	Simple and inexpensive method			
Contusion	Monitor and record impact force and duration	Weight drop model bounce control	19.4% of SCI models	Contusion forces common in SCI cases
	Reproducibility	Controlling duration of impact in non-impactor		
	Used in a wide variety of animal models	Surgical differences in clamping technique		
	Validated models			

Injuries that occur at the C level are typically incomplete injuries, as complete injuries can result in increased risk of mortality due to potential injury to areas that regulate

involuntary functions such as breathing. C SCI animal models can be used to investigate motor and sensory recovery, as well as pathophysiology post SCI as there is high clinical relevance as most SCI clinical cases are C injuries, 58% prevalence. T level injuries can be complete or incomplete, as the risk of mortality based on injury type is not as relevant in C SCI models. As mentioned earlier, a wide majority of SCI models are T SCI, 81%, with clinical prevalence around 41%. Similar to C SCI, T SCI can be used to investigate functional recovery and pathophysiology of SCI. Though far less common, L and S level SCI are utilized in animal models, making up 5% and less than 1% of studies. Lumbrosacral SCI, makes up about 10% of SCI cases within the US. Animal models using L SCI have typically use these injuries to investigate spasticity. In regards to S SCI, these injuries tend to affect the lower extremities, with S SCI primarily affecting the tail in animal models of SCI. S SCI studies are typically not used to assess motor recovery as the motor neuronal inputs to the tail differ from motor inputs to the limbs [2, 75-81].

The model utilized in both studies was an incomplete C SCI. The reasons for selecting this model was due to the high clinical relevance, the ability to assess both motor and sensory function, NP development, and histological outcomes [82-84]. Though within the human population C SCI makes most SCI cases, it only makes up slightly more than a tenth of animal studies. This disparity may be due to the elevated risk of mortality in C SCI models due to the proximity of the brainstem, as well as the comparatively more invasive surgery needed to access the C region to the T region in animals [82, 85]. The wide difference between clinical and literature cases indicate that there may be large gaps in knowledge, as level of injury has great impact on extent of dysfunction post SCI.

Stress States, Physiological Effects, and the Effects of Stress on Pain

Acute and Chronic Stress

Physiologically, the role and results of stress are dependent on the duration and amount of stress exposure. Stress exposure, acute or chronic changes to physiological homeostasis or externally perceived threats, activates the hypothalamic pituitary adrenal (HPA) axis (figure 3) [86, 87]. Activation of the HPA axis leads to the hypothalamus release of corticotropin releasing hormone (CRH) which activates the pituitary gland to secrete adrenocorticotropin releasing hormone (ACTH) into the blood stream. ACTH then activates the adrenal glands to release glucocorticoids (GC) such as cortisol (CORT) into the blood stream. CORT has a wide range of target tissue types, including the CNS where CORT acts as a negative regulator of the HPA axis, stopping the release of CRH and ACTH. CORT is able to diffuse directly into the cell, where it can then bind a GC receptor. Once bound, the complex is able to translocate to the nucleus where transcriptional activity is altered [86-88].

Acutely, the effects of stress-mediated translational activity is the increased production of anti-inflammatory compounds. However, chronic stress exposure leads to decreased production of anti-inflammatory compounds and instead causes increased production of pro-inflammatory compounds, which in the context of SCI and 2° injury could exacerbate damage caused by this cascade effect thereby leading to worsened outcomes post injury [89]. In addition to the transcription of anti-inflammatory genes, a negative feedback regulator of the GC receptor is also transcribed, FK506 binding protein 51

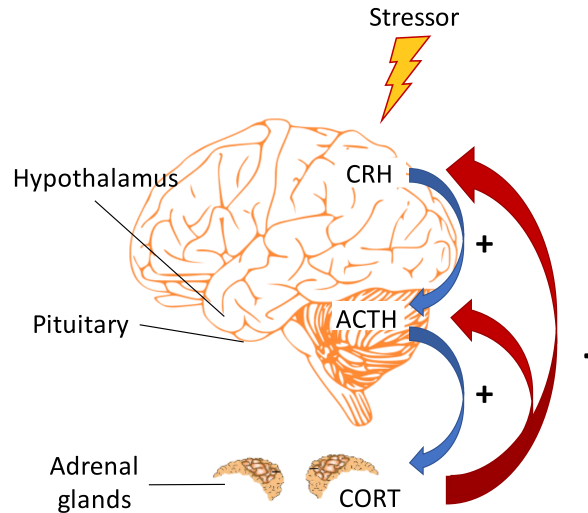


Figure 3: Activation and deactivation of the hypothalamic pituitary adrenal axis. An internal or external stressor activates the HPA axis, which activates the hypothalamus to release CRH. CRH then activates the pituitary gland to release SCTH, which then activates the adrenal glands to release of CORT. CORT feeds back into the CNS and shuts off the hypothalamus and pituitary gland, thereby turning of the HPA axis.

(FKBP51). FKBP51, a chaperone protein, allosterically binds to the GC receptor, which inhibits the binding of CORT, thereby yielding transcriptional activity and halting both the transcription of anti-inflammatory genes and FKBP51 until the HPA axis is activated again. However, in the context of chronic stress, the presence of CORT is increased and persistent, causing transcription of FKBP51 to also be increased and persistent. Therefore, the ability for anti-inflammatory compounds to be produced is decreased, causing an enhanced pro-inflammatory environment, which is observed in both sexes [90-93].

Though the overall process of stress induced HPA axis activation and transcriptional modification by CORT is similar in both males and females, sex differences exist within this pathway. It has been observed in animal models of stress, that females exhibit a

quicker activation of the HPA axis and greater release of CORT compared to males [94-97]. Considering the greater CORT released in females compared to males in response to the same stimuli and that the pro-inflammatory effects of chronic stress depends on the length and amount of CORT presence, it is important to consider the effects of sex and mode of stressor when evaluating the effects of chronic stress as there is great potential for sex differences.

Models of Stress: Modes of Stress, Animals, and Strain Differences

There are many modes for inducing acute or chronic stress in animal models, each with their advantages and disadvantages. Considering the variety of stressors and animal models, it is important to understand the traits of both. Stressors can be divided into two broad categories, physical or psychological, the result of both being activation of the HPA axis.

The decision on which type of stressor to use can be based upon aspects of the stressor such as controllability and chronicity which were factors considered in stressor selection in the research presented in this dissertation. Stressor controllability is the ability for the animal to cope with the stress stimuli. Beyond, controllability, chronicity is a key aspect in ensuring the stressor will induce chronic stress. Key aspects of stressors able to induce a chronic stress phenotype are unpredictability and sustainability. Unpredictability prevents the HPA axis from adapting to the stressor, allowing for repeated activation of the stress response pathway. Sustainability allows for the stressor to be given repeatedly, which will allow for the physiological switch from acute to chronic to occur. The exact switch from acute to chronic stress is unclear in regards to time, i.e. number of exposures, days; however it is known that increased stress duration allows for the switch to occur,

therefore unpredictability and sustainability are essential to stressor selection [88, 98-101].

The mode of stressor utilized in the research conducted in the present dissertation was inescapable foot shock (FS). This model of stress has been characterized in a wide range of studies including acute and chronic stress as well as studies investigating pain in rodents. These studies have shown that FS has reliability in inducing chronic stress via its unpredictability and sustainability [102-107]. The studies utilizing chronic, unpredictable FS found that exposure to stress prior to pain disorder development exacerbated visceral pain, and that administration of an antagonist against factors that aid in the release of CORT resulted in pain responses similar to unstressed controls [104, 106, 108]. These studies support the use of chronic, unpredictable FS as a means of examining the role of chronic stress in development of pain disorders.

Beyond stressor choice, animal characteristics are also important to consider. Sex has been shown to affect stress, as described earlier. In addition, animal strain has been shown to have effects on stress. It has been demonstrated that the release of CORT varies in different strains of rat. For example, Wistar rats are hyper-responsive in CORT secretion compared to Sprague Dawley rats and Lewis rats are hypo-responsive in CORT secretion compared to Sprague Dawley animals. Considering the importance of CORT in the physiological changes caused by stress exposure, animal sex and stain must be considered when examining the effects of stress on a given system [109-112].

Effects of Chronic Stress on SCI and Pain Disorders

Considering the focus of the research performed was evaluating and characterizing SCI-NP, the effects on chronic stress on SCI and pain disorders will be discussed. The subject of stress and SCI has primarily been examined in the context of stress after injury; specifically, stress caused by SCI and the effect on patient outcomes. To date, few studies have examined the effects of stress prior to SCI, meaning much remains unknown. The few studies that examined stress prior to injury, have examined post-traumatic stress disorder (PTSD) in veterans with SCI. In these studies, it was determined that PTSD caused by stress experiences prior to injury resulted in decreased social involvement post injury as compared to patients without PTSD. Additionally, it was found that diminished social interaction was exacerbated by level of injury, where greater functional impairment exacerbated PTSD symptoms, and increased pain outcomes [113, 114]. In models of visceral pain, it has been demonstrated that chronic stress exposure prior to pain induction exacerbates pain outcomes [104, 106, 108]. Considering that chronic stress has been shown to exacerbate pain in animal models of visceral pain and clinically it has been shown that stress exposure prior to SCI affects outcomes including pain, investigating the effects of chronic stress prior to SCI has great importance to the field of SCI and SCI-NP.

Cannabidiol: Therapeutic Potential for SCI-NP

Cannabidiol: Clinical and Experimental Treatment for NP

Cannabidiol (CBD), a substituent of *Cannabis sativa* (*C. sativa*), has been utilized in animal models of NP and clinically to treat NP to great effect. The impetus to use and characterize CBD as a treatment for SCI-NP is due to several factors such as the ability for CBD to penetrate the CNS rapidly after administration, the poor affinity and slight antagonistic activity of CBD for cannabinoid receptors which renders CBD unable to elicit the euphoric high associated with cannabinoid drugs, and the ability for CBD to exert pleiotropic and ameliorative effects on 2° injury [115-122].

A clinical study conducted by GW pharmaceuticals using a 1:1 combination of delta9-tetrahydrocannabinol (THC) and CBD in SCI patients with chronic injury found that patients who received the drug reported fewer spastic events and decreased pain although bouts of spasticity and pain events did not differ from the control group [123]. These results indicate that compounds derived from *C. sativa* have therapeutic potential in treating outcomes after SCI and necessitate further inquiry, especially regarding timing of administration post injury as aspects of 2° injury change temporally [11, 43, 44, 46]. Further discussion of CBD treatment in regards to SCI will be further discussed in subsequent chapters of this dissertation.

Significance

Globally, millions are affected by SCI, and within this patient population the vast majority go on to develop NP, a chronic pain condition that is debilitating and severely

negatively impacts quality of life. Patient demand for therapies and management strategies for SCI-NP are great, thereby necessitating the SCI field to investigate this disorder. By increasing understanding, the potential for developing a therapeutic for SCI-NP will also be increased. The goals of these studies were to 1) better understand what contributes to SCI-NP development, as SCI-NP development is not correlated to level or severity of injury, and the high variability between patients due to the accidental nature of SCI; and to 2) investigate the therapeutic potential of CBD in protecting against SCI-NP development post injury. Through these studies, we aimed to increase understanding of SCI-NP and aid the field in furthering its endeavor to not only better understand SCI and SCI-NP, but to meet the needs of SCI patients.

CHARACTERIZATION OF CHRONIC, MILD STRESS PRIOR TO SPINAL CORD INJURY IN MALE SPRAGUE DAWLEY AND LEWIS RATS

by

AMANDA MOHAIMANY-APONTE, MEREDITH T. ROBBINS, AND CANDACE L. FLOYD

In preparation for *Journal of Neurotrauma*

Format adapted for dissertation

Characterization of chronic, mild stress prior to spinal cord injury in male Sprague Dawley and Lewis rats

Amanda Mohaimany-Aponte[1], Meredith T. Robbins[2], and Candace L. Floyd[1]

[1] Department of Physical Medicine and Rehabilitation, University of Alabama at Birmingham, Birmingham, Alabama 35294 and [2] Department of Anesthesiology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Abstract

A vast majority of spinal cord injury (SCI) patients go on to develop chronic, neuropathic pain. Currently, there are no effective therapeutics for this disorder, stemming from poor understanding of underlying mechanisms of chronic pain development and maintenance. It has been shown that abnormal signaling from damaged nerves is a root cause of neuropathic pain. Stress has been shown to increase pain nociception in models of chronic pain disorders; however, no studies have characterized the effects of chronic, mild stress on development of pain after SCI. The goal of this study was to characterize strains of rats with differing stress responses on pain development post SCI. Sprague Dawley (SD) and Lewis (LEW) rats were divided into: control ± stress or SCI ± stress. Before SCI, stress groups were exposed to intermittent foot shocks (1mA) for 15 minutes for 7 consecutive days. Control animals received no foot shock. SCI groups received a hemi contusion at the fifth vertebral level. After SCI, assessments of pain and functional recovery occurred weekly for 4 weeks. Pain evaluations included thermal hyperalgesia (radiant heat paw withdrawal assessment), mechanical (von Frey) and cold allodynia (Acetone test). Motor function was assessed via dominant paw usage (cylinder test). SCI

diminished forepaw usage in SD and LEW rats, with no stress effect observed, however, SD rats had greater functional deficit compared to LEW rats. No injury or stress effects were observed in Von Frey or radiant heat paw withdrawal in both strains, however effects of injury, stress, and strain were observed in the Acetone test. SD SCI rats had increased paw withdrawals compared to controls and LEW SCI groups. In addition, stress effects were observed between SD SCI groups but not LEW groups. These findings indicate stress prior to SCI increased pain in SD but not LEW rats. In addition, LEW SCI rats had increased functional recovery compared to SD rats.

Key words: spinal cord injury; neuropathic pain; chronic, mild stress

Introduction

Chronic pain disorders affect almost one in four adults in developed nations [1]. Currently, there are few effective strategies to treat and manage chronic pain disorders, which greatly affect quality of life for these individuals [1-3]. Neuropathic pain, a chronic pain disorder caused by abnormal nerve signaling from damaged nerves, is characterized by two pain phenotypes, allodynia and hyperalgesia [3-11]. Allodynia is characterized by non-painful stimuli inducing pain, such as cold or touch [4, 10-13]. Hyperalgesia is characterized by increased pain sensation to painful stimuli, such as heat [3, 7, 14-16]. The underlying mechanisms of neuropathic pain are not well understood, revealing a great need to characterize neuropathic pain as well as animal models investigating pain in order to develop therapeutics as quality of life is negatively affected by neuropathic pain [17-21].

Neuropathic pain affects over two thirds of individuals with SCI, and patients frequently list neuropathic pain as a priority concern, often listed before functional recovery [17, 18, 21, 22]. A critical issue investigating SCI induced neuropathic pain is that development of pain is highly variable among patients and is not correlated to level or severity of injury, thereby causing difficulties in producing animal models that fully recapitulate the clinical condition [17, 23]. The underlying mechanisms are not fully elucidated, however previous studies investigating mechanisms of neuropathic pain have found that inflammation contributes to pain development, and increased inflammation at the site of damage increases incidence of pain as well increasing the spread of damage away from the site of injury [24, 25].

Considering the variability seen in neuropathic pain development in the SCI patient population, we considered that physiology prior to SCI could be contributing to the development of SCI induced neuropathic pain. Stressors, internal and/or external changes, have been shown to greatly affect physiology as the activation of the stress pathway and subsequent release of stress hormones, glucocorticoids (GC), have been shown to have a vast array of targets including the immune system, metabolism, blood pressure, organs, and the central nervous system (CNS) [26-34]. In addition to having widespread physiological effects, stress has also been shown to increase pain states in different pain disorders, such as visceral pain and neuropathic pain [12, 35-40]. Previous studies have also shown that nerve damage and cellular responses to stress, specifically chronic stress, and lead to similar outcomes. For example, both nerve damage and chronic stress have been separately shown to increase inflammation, production of reactive species, and activation of immune cells such as microglia, the resident

macrophage of the CNS [27, 37, 41, 42]. These components are also increased during the secondary injury cascade in SCI, which propagates of nerve damage out from the site of injury leading to increased nerve damage [9, 43-49]. Currently, no studies have investigated the effects of chronic, mild stress on the development of neuropathic pain after SCI. Therefore, the purpose of this study was to investigate the effects of chronic, mild stress on pain and functional recovery after SCI in strains of rats with differing levels of basal GC release.

Material and Methods

Subjects

Male Sprague Dawley (SD) and Lewis (LEW) rats, from Charles River Laboratories, 6 – 8 weeks of age were handled daily for 5 consecutive days. Animals were held and stroked from head to tail for 4 minutes each day. After handling, animals were habituated to behavioral equipment for 5 consecutive days. Animals were placed in one apparatus per day and allowed 20 minutes for habituation without any application of stimuli. After habituation and training procedures, baseline measures were taken. Post baseline assessments, animals were then transferred to a facility with the foot shock equipment, and post stress animals were transferred to their primary housing facility for surgery and subsequent behavioral evaluation. During behavioral assessments, animals were given 5 minutes prior to the task to habituate to the equipment and cease exploratory behavior. Animals were given 5 minutes between applications of stimuli during assessments to prevent cross reactivity between stimuli. Animals were exposed to one behavioral

assessment per day during the light cycle. Post-surgery, animals were weighed daily. At 30 days post-surgery, animals were euthanized and tissue was collected for histological analysis.

Animals were randomly assigned into stress (S) or control. After stress paradigm exposure, animals were randomly assigned into injury (SCI) or control (laminectomy; LAM) groups and coded under a new identification number. The investigator performing surgery and behavioral assessments was blinded group placement of the animal. Four groups were assessed: LAM, LAM S, SCI, and SCI S. SD sample sizes were: LAM n = 11, LAM S n = 11, SCI n = 13 and SCI S n = 11. LEW sample sizes were: LAM n = 12, LAM S n = 12, SCI n = 13, and SCI S n = 13.

Throughout the duration of the study, animals were group housed 2 – 3 animals per cage with *ad libitum* access to food (Harlan Laboratories, 7917 NIH-31 Irradiated Open Formula Mouse/Rat Diet) and water in a temperature, humidity, and light controlled facility (12:12 light/dark). All procedures performed were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and in conjunction with the National Institutes of Health guidelines on animal research.

Stress Paradigm: Inescapable foot shock

All animals were placed individually in operant chambers encased in sound and light attenuating housing 15 minutes once per day for 7 consecutive days. Animals receiving stress were exposed to 30 foot shocks (1 mA) for a duration of 1 second during the 15 minute interval. Animals in the non-stress group did not receive any foot shock [50]. After operant chamber exposure, the number of fecal matter pellets in the chamber was

counted. In addition to counting fecal matter pellets post operant chamber exposure, fecal matter pellets were collected during baseline measures, on day 7 of induction of the stress paradigm, and weekly for 4 weeks post-surgery. Fecal matter pellets were stored in -20°C until use in a corticosterone ELISA assay (Cayman Chemical, Corticosterone EIA kit).

Corticosterone Assay

Collected fecal matter pellets were dried for 8 days in a 60°C oven and then ground into a fine powder using a mortar and pestle. 50 mg of this powder was placed in a tube and 1 mL of 80% methanol was added. The mixture was then agitated for 30 minutes using a shaker. After, the tubes were placed in a centrifuge and spun for 20 minutes at 2500 x g. The supernatant was collected and placed in a separate tube, the pellet was discarded. The supernatant was then allowed to evaporate, leaving the extracted residue in the tube. The dried sample was then suspended in 200 µL of EIA buffer. EIA buffer was prepared by using 1 vial of EIA buffer 10X (supplied by the kit) in 90 mL of ultrapure water.

Reagents for the kit were prepared as well. 100 µL of CORT EIA standard (bulk standard) was placed into a tube and diluted with 900 µL of ultrapure water. Eight serial dilutions were made, the first serial dilution with 900 µL of EIA buffer and the subsequent dilutions with 750 µL of EIA buffer. 100 µL of the bulk stand was added to serial dilution 1 and mixed. 500 µL of this solution was then transferred to serial dilution 2, and mixed. 500 µL of this solution was then added to serial dilution 3, and mixed. This continued until 8 serial dilutions were made. CORT AChE tracer was made by adding 100 dtn CORT AChe tracer to 6 mL EIA buffer. CORT EIA antiserum was made by adding 100 dtn CORT antiserum to 6 mL EIA buffer. Wash buffer was prepared by

adding 195 mL of ultrapure water to 5 mL of wash buffer 400x concentrate. After sample, EIA buffer, wash buffer, and reagent preparation the assay was then ready to be run.

100 µL of EIA buffer was added to the non-specific binding wells in duplicate and 50 µL the maximum binding wells in triplicate. The serial dilutions were added in duplicate to their respective wells. 50 µL of the fecal matter samples were then added in triplicate to their respective wells. 50 µL of CORT AChE tracer was added to each well except the blank and total activity wells. 50 µL of CORT EIA antiserum was then added to each well except the blank, total activity, and non-specific binding wells. The plate was then covered with plastic film and incubated for 2 hours at room temperature on an orbital shaker. After incubation, the assay was ready to be developed.

Ellman's reagent was mixed with 20 mL of ultrapure water and shielded from light exposure with foil. The wells were emptied and rinsed 5 times with wash buffer. After the washes, 200 µL of Ellman's reagent was added to each well in a dimly lit room. 5 µL of CORT AChE tracer was added to the total activity well. The assay was then covered with plastic film and covered in foil to ensure shielding from light, and placed on an orbital shaker for 60-90 minutes. Post Ellman's reagent incubation, the assay was ready to be read.

The plate was removed from the shaker and the coverings were gently removed to prevent any spillage of Ellman's reagent from the wells. The plate was then read using a microplate reader (Synergy H1, BioTek) at a wavelength between 405 and 420 nm (412 nm specifically recommended by the kit). If absorbance of the maximum binding wells

exceeded 2.0 A.U. then the plate was washed and fresh Ellman's reagent was added and incubated again. If the absorbance of the maximum binding wells was between 0.3 A.U. and 1.0 A.U. then the plate did not need to be washed and redeveloped.

The absorbance information was imported into an Excel spreadsheet (Microsoft) and the data was prepared by separately averaging the absorbance from the non-specific binding wells and the maximum binding wells. These averages were then subtracted from one another giving the corrected maximum binding value. The sample amount bound over the corrected maximum binding was determined by subtracting the non-specific binding average from the sample and divided by the corrected maximum binding value giving the sample concentration. This was repeated for all the sample values. These values were multiplied by 100 to give the percentage of sample concentration. The data was plotted as the sample concentration versus the log concentration using a linear regression fit.

Surgery

Animals were anesthetized with a 4% isoflurane in oxygen for 4 minutes, and maintained with 2% isoflurane in oxygen. The area of incision, dorsal portion of the neck to shoulder blades, was shaved and cleaned with betadine and chlorohexidine. Heating pads were used to maintain a body temperature of 37°C±1°C; anal probes were used to assess body temperature throughout the entire surgical procedure. After toe pinch to ensure anesthesia, an incision was made from the dorsal region of the neck to the shoulder blades. Incisions were made through subsequent muscle layers to expose spinal vertebrae from cervical level 2 (C2) to thoracic level 2 (T2). The spinal process of C5 was dorsally laminectomized to reveal the spinal cord. Animals assigned into the SCI group received a

300 kdyne impact with 0 dwell time (Infinite Horizon SCI Device, Precision Systems and Instrumentation; Lexington, Kentucky) on one side of the spinal cord, resulting in an incomplete injury. The side of impact was determined via dominant forepaw, meaning the dominant forelimb was ipsilateral to injury. Animals in the control group received no impact [51]. After all surgical procedures were completed, animals were sutured. Muscle layers were sutured with a polydioxanone synthetic absorbable sterile suture (Monosorb ™), the skin layer was sutured using a polypropylene non-absorbable sterile suture (Butler Schein ™). Post suturing, animals were given a subcutaneous injection of 5 mg/mL carprofen (dosed at 1 mL per kg), 0.2 mL antibiotic (Bayer Healthcare LLC, Baytril ®, enrofloxacin), and 3 mL saline. Animals were then placed in an incubation cage and observed until ambulation. Sutures were removed 7 days post-surgery.

Behavioral and Functional Assessments

Unskilled forelimb function: Paw Placement and Dominant Paw Determination

Animals were placed in a Plexiglas cylinder (40 cm height, 30 cm diameter) for 5 minutes. During which time the number of forepaw placements on the wall of the cylinder was tallied as either right, left, or both. Paw placements in which there was full paw placement with body support were tallied. Right and left placements indicate placement of either the right or left forepaw. A tally of both indicates indistinguishable and simultaneous placement of both forepaws upon the wall. The percent of right, left, or both placements can be determined using this equation: (right or left or both numbers of placements)/ (sum of right, left, and both placements)*100. Number of rears were also calculated from each paw placement session post-surgery. From the baseline

measurements, the dominant paw of the animal was determined. Dominant paw was defined as right or left forepaw use 50% or greater, determined by using 25 or more total paw placements. Dominant paw was determined prior to surgery.

Thermal Hyperalgesia: Radiant Heat Paw Withdrawal Assessment

Animals were placed on a Plexiglas platform in individual Plexiglas chambers (21.5 cm length x 12.5 cm width x 12.5 cm height). Under this platform, a maneuverable light source (Ugo Basile Plantar Test, Biological Research Apparatus) was able to be positioned under the plantar region of the hind paws, a mirror was utilized to ensure full visibility of the animal. After placement of the light source, the light source was activated. As time passed, the intensity of the light increased. The latency to paw withdrawal was measured in seconds, with a cutoff time set at 30.1 seconds to prevent tissue damage. Each hind paw was assessed 3 separate times on each assessment day. The average latency to paw withdrawal using these 3 times was calculated.

Mechanical Allodynia: Von Frey

Animals were placed in individual Plexiglas chambers (21.5 cm length x 12.5 cm width x 12.5 cm height) on a platform with a wire mesh bottom. A Von Frey filament (Touch Test ®, North Coast Medical and Rehabilitation Products) was applied perpendicularly to the plantar region of the hind paw for 3 – 5 seconds with enough force to slightly bend the filament five separate times. The starting filament was set at 2 grams for all animals and the terminal filament set at 15 grams. The up/down method was employed, if the animal had 3 or more withdrawal responses to a filament, the next filament used was one with a smaller force unit. If the animal had less than 3 withdrawal responses, a filament

of greater force was used. This up/down paradigm continued until the terminal filament was used without 3 or more withdrawal responses or the same filament had 3 or more positive responses on 2 separate application events. The 50% paw withdrawal threshold, Dixon score, was calculated using the following equation: Xf + kd, Xf: final filament force, k: constant; average difference of force between filaments, d: tabular value based on pattern of paw responses.

Cold Allodynia: Acetone Test

Animals were placed on a platform with wire mesh bottom in individual Plexiglas enclosures (21.5 cm length x 12.5 cm width x 12.5 cm height). A bubble of acetone was applied to the plantar region of the hind paw without mechanical force and allowed to evaporate in order to elicit a cooling sensation. The animal was observed for 20 seconds post acetone application for a paw withdrawal response. Each hind paw was assessed five separate times. The percent of paw withdrawal incidence was calculated from these values.

Histology

Tissue Collection and Preparation

On day 30 post-surgery, animals were deeply anesthetized with isoflurane and perfused intracardially with cold, 0.1 phosphate buffered saline (PBS) at a pH of 7.4 for 5 minutes. Animals were then perfused with fixative (American MasterTech Scientific, Inc. ExCell PLUS ™). The cervical spinal cord area was then removed and post fixed for 5 days at 4°C. Tissue was then placed in a 10% sucrose PB solution for one hour at 4°C, and then placed in a 30% sucrose PB solution for 48 hours at 4°C. The cervical tissue was then

sectioned into three pieces three millimeters in size, one section at the lesion epicenter (C5), one section caudal to the epicenter, and one section rostral to the epicenter. The sections were then placed in an embedding medium and stored in a -80°C freezer until slicing and slide mounting. Glass slides were coated with 1% gelatin and chrom alum. Traverse, serial 30 µm slices were made using a cryostat machine (Leica Biosystem CM1860) and thawed onto gel coated glass slides. Slices were mounted in series of tens meaning a 300 µm interval between adjacently mounted slices, with each slide representing 2100 µm of tissue centered at the epicenter of the injury site (C5). Slides were stored at -20°C until staining. After staining, slides were evaluated using an Olympus IX73 microscope with Visiopharm® imaging software.

Neuronal Numbers: Cresyl Violet

After tissue had been slide mounted, the slide was subjected to dehydration, defatting, rehydration, cresyl violet staining, differentiation, and dehydration. The tissue was dehydrated by being placed in ethanol baths of increasing ethanol concentrations, 75%, 95%, and 100%, for 2 minutes. Defatting occurred by exposing the dehydrated tissue to three xylene baths at 5 minutes, 10 minutes, and 1 minute. After defatting, the tissue was rehydrated by re-exposing the tissue to ethanol in decreasing order of concentration, 100%, 95% and 70% for 1 minute and then two deionized water baths for 30 seconds. The tissue was stained with cresyl violet acetate for 2 – 10 minutes, depending on the thickness of the tissue and the age of the staining medium. After the cresyl violet acetate bath, the tissue was rinsed in two deionized water baths for 15 seconds. The tissue was then placed in 95% ethanol for 2 – 10 minutes, depending on cresyl violet staining time and tissue thickness, with acetic acid to differentiate staining of non-nissl substances

from nissl. The tissue was then dehydrated by being exposed to ethanol baths, 95% and 100% concentrations, for 30 seconds and then two xylene baths for 5 minutes. After the second xylene bath, the slide was coverslipped with mounting media.

Myelin Sheath Integrity

After slide mounting the tissue, the sample was dehydrated, defatted, rehydrated similar to the cresyl violet stain. After defatting, tissue was placed in a 0.2% eriochrome cyanine solution for 10 minutes and then placed in two water baths for 30 seconds each. The tissue was then differentiated by being placed in a 0.5% ammonium hydroxide bath for 1 minute and then two water baths for 30 seconds each. Post differentiation, the tissue was dehydrated similar to the cresyl violet stain protocol and coverslipped with mounting media.

Data Analysis

Data was analyzed using statistical software GraphPad Prism 6.0. The behavioral data shown are means ± standard error. Significance was set at $p \leq 0.05$, * indicates significance between injured (SCI) and control (LAM) groups, + indicates significance between stress (S) and non-stress control groups, and ǂ indicates significance between strains (SD and LEW).

Results

Effects of SCI on Neuron Counts and White Matter Volume

At four weeks post-surgery, animals were euthanized and cervical vertebral spinal cord tissue was collected for histological analysis. In both SD and LEW animals, SCI decreased the number of neurons present at the epicenter of injury (Figure 1A, SD: $F_{(1,30)}$=28.43, $p< 0.0001$; LEW: $F_{(1,32)}$= 32.18, $p < 0.0001$). In addition, it was observed that the area ipsilateral to injury had significantly less neurons present compared to the area contralateral to injury (Figure 1A, SD: $F_{(1,16)}$=34.57, $p < 0.0001$, LEW: $F_{(1,16)}$= 35.45, $p < 0.0001$). No effect of strain or stress was observed on neuronal numbers (Figure 1A). White matter volume was significantly diminished at the epicenter of injury in SCI animals regardless of strain or stress exposure (Figure 1B, SD: $F_{(1,30)}$= 99.7, $p< 0.0001$; LEW: $F_{(1,30)}$= 142.7, $p < 0.0001$). It was also observed that white matter volume was significantly decreased ipsilateral to injury (Figure 1B, SD: $F_{(1,16)}$= 129.2, $p < 0.0001$; LEW: $F_{(1,16)}$= 191.9, $p < 0.0001$). Representative images, taken at 4x magnification, of each group shows intact histology for surgical control animals and containment of injury solely to the ipsilateral side of injury through cresyl violet staining for nissl substance (Figure 1C).

Effects of Injury, Stress, and Strain on Body Mass

Prior to surgery, all animals were weighed in order to obtain a baseline (BL) mass (g) value. After surgery, days 1 through 30 post operation (p.o.), animals were weighed once daily. The masses obtained were compared as percent change from BL for all groups. SCI significantly affected gains in mass compared to their respective control groups in SD

rats but not LEW rats (Figure 2 A and B, $F_{(3,1293)}= 122.1$, $p< 0.0001$). SD animals that received stress prior to SCI had a longer duration of diminished gains compared to their control group, day 2 – 30 p.o., than non-stressed SCI animals, day 2 – 12 and 14 p.o. (Figure 2A). There was no significant interaction between SCI S and SCI groups for both SD and LEW animals (Figure 2 A and B). At day 30 p.o., SD LAM S animals had a greater gain in body mass compared to their LEW non-stressed control counterparts (Figure 2 D, $p < 0.0001$). There was no significant difference between any other SD and LEW group upon strain comparison (Figure 2 C and D).

Effects of Stress Exposure on Fecal Pellet CORT Concentrations and Fecal Pellet Counts

Baseline measures of fecal pellet CORT show that SD animals had significantly greater CORT compared to LEW animals (Figure 3 A, $t= 2.697$, $df = 22$, $p = 0.0392$). On the last day of stress induction (day 7), fecal CORT concentrations were assessed in both LEW and SD animals that were exposed to stress (S) and non-stress paradigms. There were no significant differences observed between SD groups, however, LEW S animals had significantly greater CORT measurements compared to both LEW controls (Figure 3B, $t = 2.573$, $df = 34$, $p = 0.0450$) indicating lower concentrations of CORT. SD controls trended towards greater CORT compared to LEW controls (Figure 3B, $t = 1.347$, $df = 34$, $p = 0.0935$). Regardless of strain, exposure to foot shock increased the amount of fecal matter pellet defecation compared to non-stressed controls (Figure 3 C, $F_{(3,546)}= 1151$, $p < 0.0001$). This difference was observed on all days of foot shock exposure, days 1 through 7 ($p < 0.0001$).

Effects of Injury and Strain on Unskilled Forelimb Function: Paw Placement

Prior to surgery, all groups, regardless of strain, had equivalent dominant forelimb usage of about 60% of total paw placements. After injury, all SCI groups, regardless of strain had a significant decrease in dominant forelimb usage, limb ipsilateral to injury (Figure 4A and B, SD: $F_{(3,210)} = 178.5$, $p < 0.0001$; LEW: $F_{(3,230)} = 12.95$, $p < 0.0001$). No effect of stress was observed in either strain of rat or in non-injured control groups. This functional deficit persisted from week 1 to 4 p.o. in SD rats and week 1 and 2 p.o. for LEW rats. LEW rats recovered functional ability similar to their respective LAM groups after week 2. After comparing SD and LEW SCI groups, a significant effect of strain was observed. SD SCI rats had greater functional deficit than LEW SCI rats, which was observed in all weeks p.o. for both stressed and non-stressed SD SCI animals (Figure 4C and D, S: $F_{(1,110)} =$ $p < 0.0001$; NS: $F_{(1,120)} = 45.24$, $p < 0.0001$). Number of rearing events were not affected by injury or stress status, with no effect of strain observed (Figure 4 E and F).

Effect of Strain on Thermal Hyperalgesia: Radiant Heat Paw Withdrawal Assessment

BL and p.o. weekly assessments of latency (s) to contralateral hind paw withdrawal was measured after exposure to a heat source. No significant effect of injury or stress was observed in both SD and LEW rats. However, a strain difference was observed in week 2 p.o. between SD and LEW LAM S animals. SD rats had a greater time to withdrawal compared to LEW rats (Figure 5D, $p = 0.0337$). This difference was not observed in any other time point.

No Effect of Injury, Stress, or Strain on Mechanical Allodynia: Von Frey

Prior to surgery and weekly for 4 weeks p.o., animals were assessed on sensitivity to Von Frey filaments. The up/down method was used; if an animal showed no sensitivity to the filament used, a stiffer filament with a greater force (g) was used. If the animal did show sensitivity, a thinner filament with less force was applied. At BL and throughout the duration of the study, there was no significant effect of injury, stress, or strain on filament sensitivity.

Effect of Injury, Stress, and Strain on Cold Allodynia: Acetone Test

Similar to the other sensory tasks, animals were assessed for cold allodynia prior to surgery and for 4 weeks after surgery. The number of contralateral hind paw withdrawals after acetone exposure were converted into the percent of withdrawals during the weekly Acetone test assessment. From weeks 2 to 4 p.o., there was a significant effect of injury of the percent of withdrawals in SD rats, in addition there was no difference between uninjured controls at any time point ($F_{(3,210)} = 45.75$, $p < 0.0001$). In addition to the injury effect, SD rats that were exposed to stress prior to SCI had a greater percentage of paw withdrawal compared to SD SCI animals. This stress effect was observed in week 3 and 4 after injury ($p = 0.0438$). No effect of injury or stress was observed between LEW groups. Strain differences were observed between stress groups. SD SCI S had a greater percentage of paw withdrawal compared to LEW SCI S animals from week 1 through 4 p.o. ($F_{(3,215)} = 30.45$, $p > 0.0001$). In addition to SCI S strain differences, LEW LAM S animals had a greater percentage of paw withdrawals compared to SD LAM S animals ($F_{(1,105)} = 10.32$, $p = 0.0017$). Non-stressed groups also exhibited strain differences

($F_{(3,220)}$= 12.27, $p < 0.0001$). Similar to LAM S groups, LEW LAM animals had a greater percentage of paw withdrawals compared to SD LAM animals from weeks 2 to 4 p.o ($p = 0.0032$, $p < 0.0001$, $p = 0.00018$, respectively).

Discussion

These results show that the C5 hemi contusion model used in this study diminishes neuronal cell numbers and decreases white matter volume ipsilateral to the epicenter of injury in both SD and LEW animals. In addition, it was observed that this injury model diminished motor function of the forepaw ipsilateral to injury in both SD and LEW animals. However, this deficit was shown to persist chronically for SD animals and not LEW animals. It was also observed that SCI diminished body mass gains in SD animals and not LEW animals. Beyond this, in SD animals, this injury model caused increased incidence of paw withdrawal to non-noxious stimuli at chronic time points, as evidenced by the Acetone test. It was also found that exposure to chronic, mild stress prior to SCI exacerbated the incidence of paw withdrawal to cold stimuli in SD rats and not LEW rats.

In regard to stress, we observed that SD and LEW animals have differing basal fecal CORT concentrations, with LEW animals having higher levels of CORT in their feces. In response to stress, both SD and LEW animals had significantly increased amounts of defecation compared to non-stress controls indicating HPA axis activity. Feces analyzed on the last day of the stress paradigm showed no stress effect in SD animals as both stressed and control animals had similar CORT concentrations and a stress effect in LEW animals, with stressed groups having greater CORT levels compared to control animals.

Stress effects were observed in body mass gains in SD animals, with SCI S animals have a longer duration of diminished gains compared to SCI non-stressed controls. Beyond this, it was observed that SD SCI S animals had greater incidence of paw withdrawal in response to the Acetone test compared to non-stressed SD controls and their respective LEW SCI S group.

Overall, these data indicate that SD animals are a better strain to assess chronic outcomes post C5 hemi contusion SCI, specifically motor function, and sensitivity to cold stimuli compared to LEW animals though both showed equivalent neuronal cell loss and diminished white matter volume. In addition, this study showed modest effects of chronic, mild stress on diminished gains in body mass and sensitivity to cold stimuli in SD SCI animals. Taken together, the C5 hemi contusion SCI model in SD rats reflects outcomes observed clinically such as chronic motor dysfunction and sensitivity to non-noxious stimuli; and with exposure to chronic, mild stress having modest increase sensitivity of paw withdrawal in SD SCI animals.

Previous studies looking at outcomes post SCI comparing both SD and LEW animals have also shown strain differences in functional recovery, development of pain, and genes expressed after injury [52-54]. A previous study characterizing histological outcomes post complete thoracic SCI in female LEW, SD, and Fischer 344 (F344) rats found that LEW animals had greater lipid peroxidation compared to both SD and F344 animals in both control and SCI groups. In addition, it was found that LEW SCI animals had lower labelling of rubrospinal tract neurons and greater motor impairment compared to both SD and F344 animals [52]. Our study found that both SD and LEW animals had decreased neuronal numbers and diminished white matter volume post SCI with no

observed strain difference, and that SD SCI animals had greater functional deficit than LEW SCI animals. It is important to note that the aforementioned study utilized a different SCI model as well as female animals, indicating important effects of model and sex on outcomes post SCI. Previous studies investigating the effects of female sex hormones on outcomes post SCI have found estrogen and estradiol to be protective post injury [55-59].

Additionally, a study investigating sex effects on motor function post SCI in both SD and LEW animals found that male animals had worsened motor function post injury in both SD and LEW animals [60]. The purpose of employing the C5 hemi contusion SCI model in male animals for this study is based upon emulating the clinical SCI population. The location and impact of the SCI mirrors the most common type of SCI, incomplete tetraplegia which is estimated to make up 45% of SCI cases in the United States [61]. Beyond this, males constitute the vast majority of SCI patients, roughly 80% [62]. These results highlight the need to characterize different models of SCI and sexual dimorphism on SCI outcomes.

In addition to strain differences, we also found that exposure to chronic, mild stress prior to SCI moderately increased paw withdrawal in response to non-noxious cold stimuli in SD SCI animals, but had no effect on LEW animals. Previous studies have shown that SD rats have greater secretion of CORT compared to LEW animals, which was observed in the baseline fecal pellet CORT analysis. After stress exposure (days one through seven), it was found that both SD and LEW animals exposed to stress had increased defecation events. Stress induced defecation is a marker of the autonomic nervous system being activated [63]. The autonomic nervous system is activated by the HPA axis

therefore increased defecation post stress exposure allows for non-invasive determination of HPA axis activation. On the last day of stress exposure, day seven, fecal pellet CORT analysis showed stress exposure increased CORT secretion in LEW animals but not SD animals. In addition the baseline CORT differences observed were no longer significant, though a trend was present. Interestingly, SD animals exposed to stress were still defecating at increased amounts compared to non-stressed controls and quantities similar to their respective LEW counterparts.

Previous studies investigating the effects of stress on CORT release have found that chronic stress has the potential to attenuate and diminish CORT secretion through decreased CRH mRNA levels and receptor binding capacity in the brain, which could potentially explain why stress effects were observed in SD animals such as increased defecation post stress exposure and worsened behavioral outcomes post SCI with no fecal pellet CORT differences at the last day of the stress paradigm [26, 28, 64-66]. These results highlight the need to further explore the mechanisms behind the switch from acute to chronic stress, chronic stress effects in animal models of pain.

Looking at the literature, it is important to note that the chronic stress paradigm utilized can have varying effects. In addition to type of stressor, sex can affect response to stress [67-71]. The stressor used in this study was inescapable foot shock. Past studies have shown that this paradigm has been effective at inducing a chronic stress state, as well as exacerbating visceral pain nociception in female rats [36, 50, 72]. These studies exhibit robust effects of chronic foot shock on pain, whereas in the study presented here, only moderate effects were observed. This study utilized male animals, as males make up 80% of the SCI patient population, which have lower basal CORT concentrations and slower

CORT release in response to stress compared to females [68-71]. All together this indicates that females are more susceptible to stress, and that use of the chronic foot shock paradigm elicits robust effects on pain measures in females whereas in males using the same paradigm parameters only produced modest effects. A potential future study could be to extend the duration of the inescapable foot shock paradigm used and determine if more robust effects of stress on outcomes post SCI, specifically neuropathic pain, are observed.

Keeping SCI induced neuropathic pain in mind, a limitation of this study is that no supraspinal mechanism was utilized; meaning the responses to acetone may be reflexive rather than development of cold allodynia. A previous study investigating complete thoracic SCI and the outcomes of supraspinal versus spinal mediated responses found that hind limb paw withdrawals to stimuli post SCI were spastic, hyper reflexive responses [73]. Therefore, considering this, the paw withdrawals in response to acetone observed in SD animals have been described as a sensitization to cold stimuli rather than development of SCI induced neuropathic pain.

In conclusion, this study presents evidence of modest effects of chronic, mild stress on SD SCI animals, specifically diminished gains in body mass and paw withdrawal after acetone exposure which was not observed in LEW rats. Additionally, LEW rats had increased functional recovery post SCI though both SD and LEW animals had similar neuronal counts and white matter volume at the epicenter of injury. Through this characterization, we have found that inescapable, chronic foot shock had moderate effects on SD animals, but not on LEW animals regarding outcomes post SCI. These findings

further highlight the need to understand the model and strain of rodent being utilized, chronic stress paradigm, and SCI clinical modelling in the stress response.

Acknowledgements

The work performed was supported by the UAB Comprehensive Neuroscience Center Strategic Plan Pilot Award (CF, MR) and the TJ Atchinson Spinal Cord Injury Research Program. The graduate student performing the work was supported by the Howard Hughes Med to Grad Initiative Fellowship and the Equity and Diversity Enhancement Program.

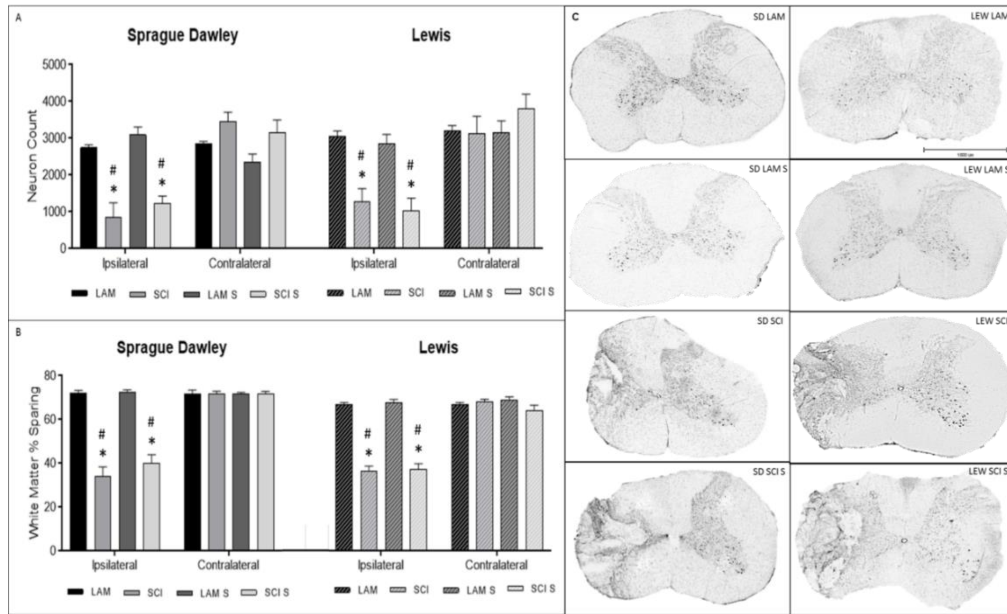


Figure 1: Spinal cord injury significantly decreases neuronal numbers and white matter at the epicenter of injury and is not affected by stress. Neuron counts were significantly decreased ipsilateral to injury in SCI groups regardless of strain or stress condition. No effects of injury were observed contralateral to injury, nor were strain and stress effects (A). White matter volume was significantly decreased ipsilateral to injury in SCI animals compared to their respective control groups (B). Representative images (4x magnification) from each group stained with cresyl violet (C). Data shown are means ± s.e. analyzed via two-way ANOVA with significance set at $p \leq 0.05$. *: significance between SCI and LAM groups, #: significance between ipsilateral and contralateral. SD sample sizes: LAM n = 11, LAM S n = 11, SCI n = 13 and SCI S n = 11. LEW sample sizes: LAM n = 12, LAM S n = 12, SCI n = 13, and SCI S n = 13.

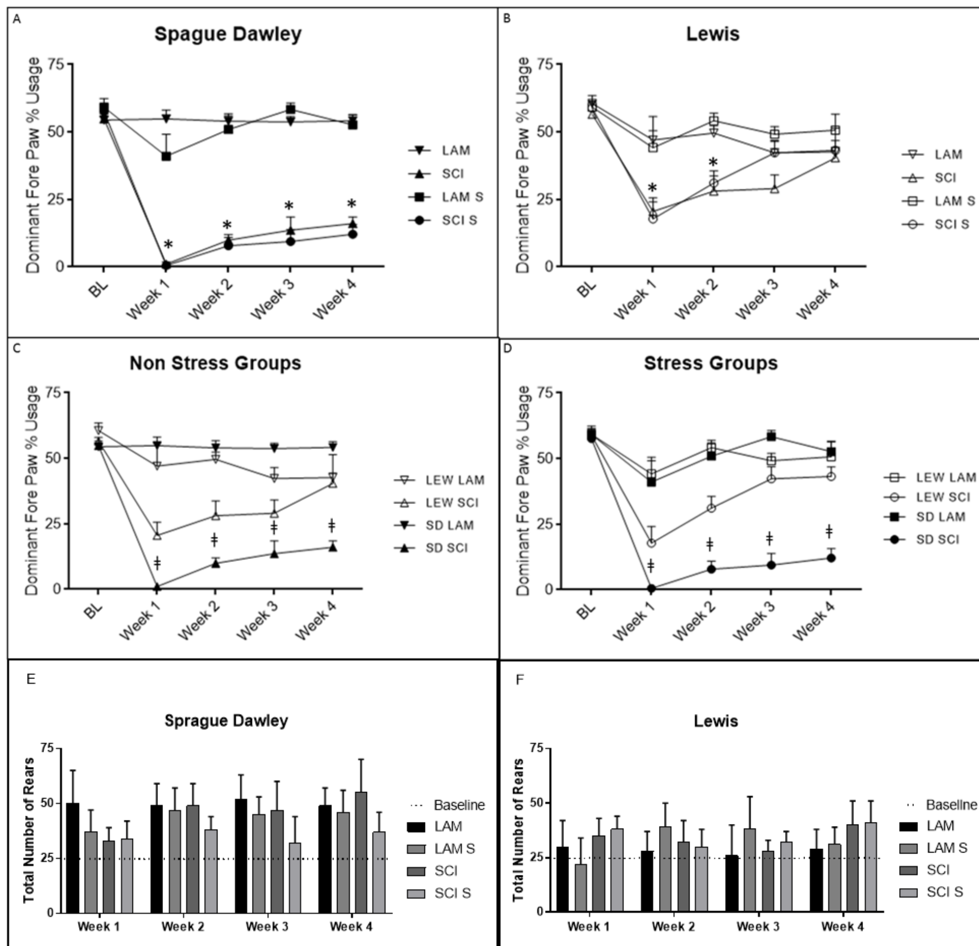


Figure 2: Injury and rodent strain, but not stress exposure, affect motor function. SCI significantly decreased dominant forepaw use (paw ipsilateral to SCI) which persisted the duration of the study (A). No effect of stress was observed on functional outcomes in both SD and LEW rats (A, B). SCI significantly decreased dominant forepaw use in weeks 2 and 3, but were able to functionally recover similar to LAM controls at weeks 3 and 4 post SCI (B). Strain differences were observed in both SCI S and SCI animals. SD SCI rats had greater functional deficit than LEW SCI rats which lasted from week 1 until week 4 (C, D). Number of rearing events at each paw placement session showed no change in animal activity, with no effect of injury, stress, or strain observed (E, F). Data shown are means ± s.e. analyzed via two-way ANOVA with significance set at $p \leq 0.05$.

*: significance between SCI and LAM groups. ǂ: significance between SD and LEW groups. SD sample sizes: LAM n = 11, LAM S n = 11, SCI n = 13 and SCI S n = 11. LEW sample sizes: LAM n = 12, LAM S n = 12, SCI n = 13, and SCI S n = 13.

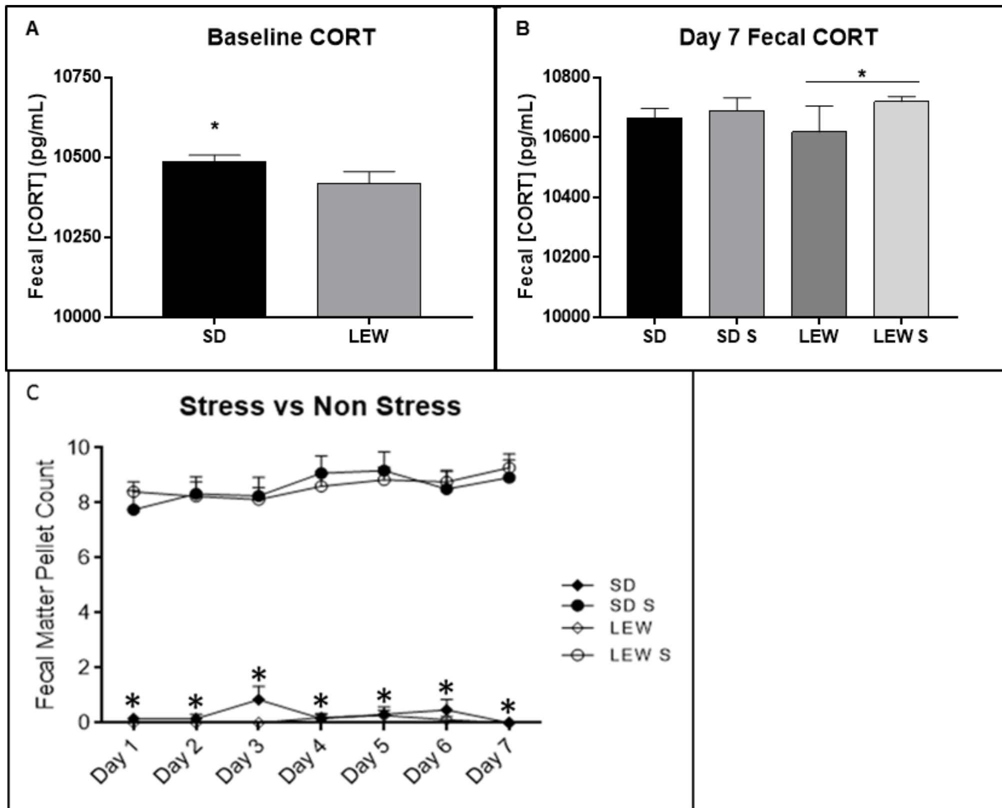


Figure 3: Stress differences between strains and the effects of stress exposure. Prior to induction of stress, LEW animals had significantly lower amounts of basal CORT present in fecal matter pellets compared to SD animals (A). On the last day of stress induction, LEW control animals had significantly less fecal CORT compared to LEW S (B). No significant differences were observed between SD S and control groups (B). Exposure to stress increased the amount of defection in SD and LEW rats from day 1 to day 7 of operant chamber exposure. No effect of strain was observed on amount of defecation (C). ELISA data shown are means ± s.e. analyzed via t-test with significance set at $p \leq 0.05$. *: significance between SD and LEW groups. Baseline SD and LEW n = 12, assayed in triplicate. ELISA day 7 SD and LEW n = 6 per group, assayed in triplicate. Fecal pellet count data shown are means ± s.e. analyzed via two-way ANOVA with

significance set at $p \leq 0.05$. *: significance between S and NS groups. SD S n = 12, SD NS n = 19, LEW S n = 25, LEW NS n = 26.

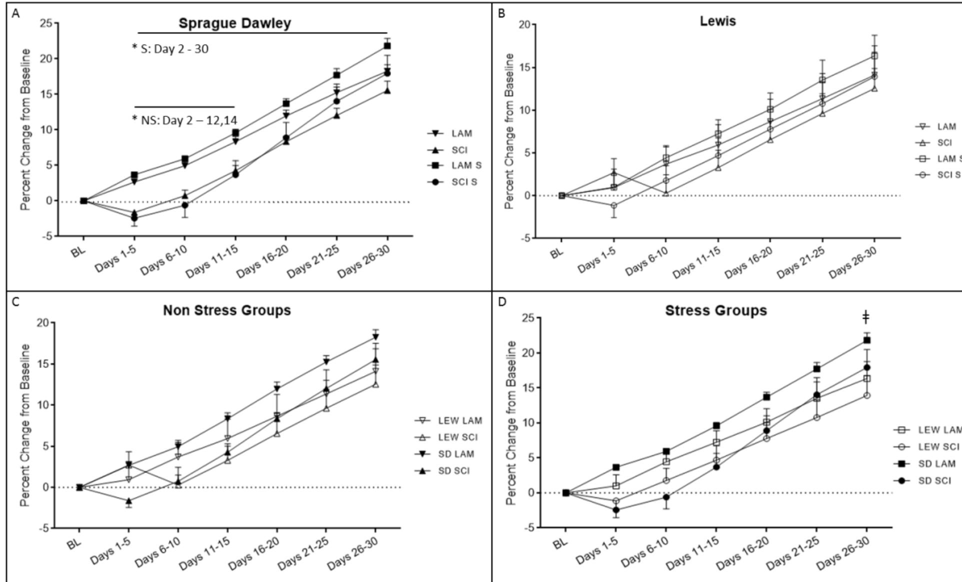


Figure 4: Body mass gains are affected by injury and strain. Percent changes in body mass (g) gains were diminished in SD rats that received a SCI (A), but not in LEW SCI rats (B). SD SCI S rats had a longer duration of diminished gains, day 2 to 30 p.o., compared to SD SCI rats, day 2 to 12 and 14 p.o. (A). In addition, at day 30 p.o., SD LAM S rats had greater gains in body mass than LEW LAM S indicating strain differences (C). No differences were observed in strain comparison between non-stressed groups (D). Data shown are means ± s.e. analyzed via two-way ANOVA with significance set at $p \leq 0.05$. *: significance between SCI and LAM groups. SD sample sizes: LAM n = 11, LAM S n = 11, SCI n = 13 and SCI S n = 11. LEW sample sizes: LAM n = 12, LAM S n = 12, SCI n = 13, and SCI S n = 13.

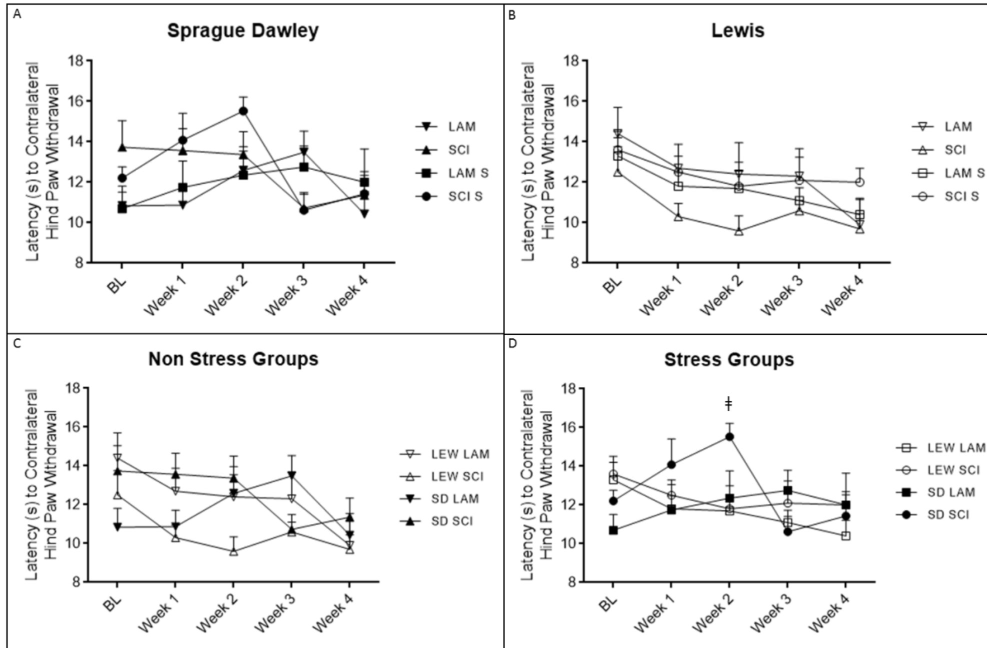


Figure 5: Thermal hyperalgesia is not affected by injury or stress exposure, with intermittent effects of strain. There was no significant effect of SCI or stress exposure in either SD or LEW animals (A, B). At week 2 p.o.. There was no strain difference observed in non-stressed SD and LEW animals (C). SD SCI S animals had a higher latency to paw withdrawal compared to LEW SCI S animals (D) Data shown are means ± s.e. analyzed via two-way ANOVA with significance set at $p \leq 0.05$. ǂ: significance between SD and LEW groups. SD sample sizes: LAM n = 11, LAM S n = 11, SCI n = 13 and SCI S n = 11. LEW sample sizes: LAM n = 12, LAM S n = 12, SCI n = 13, and SCI S n = 13.

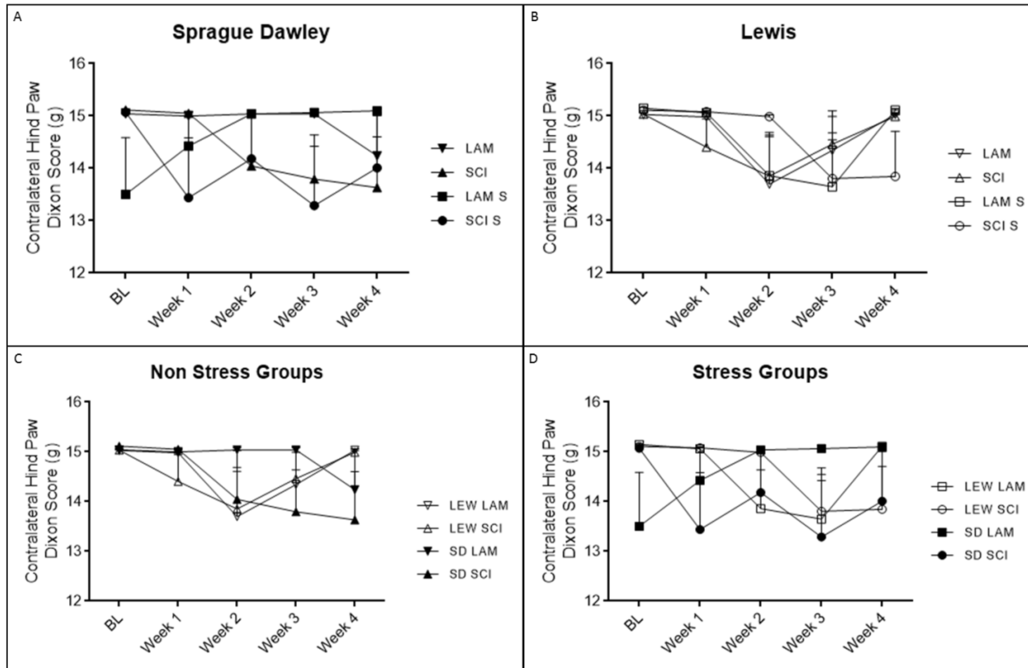


Figure 6: Mechanical sensitivity is not affected by strain, stress exposure, or injury. There was no significant effect of SCI, stress, or strain on sensitivity to Von Frey filaments (A, B, C, D). Data shown are means ± s.e. analyzed via two-way ANOVA with significance set at $p \leq 0.05$. SD sample sizes: LAM n = 11, LAM S n = 11, SCI n = 13 and SCI S n = 11. LEW sample sizes: LAM n = 12, LAM S n = 12, SCI n = 13, and SCI S n = 13.

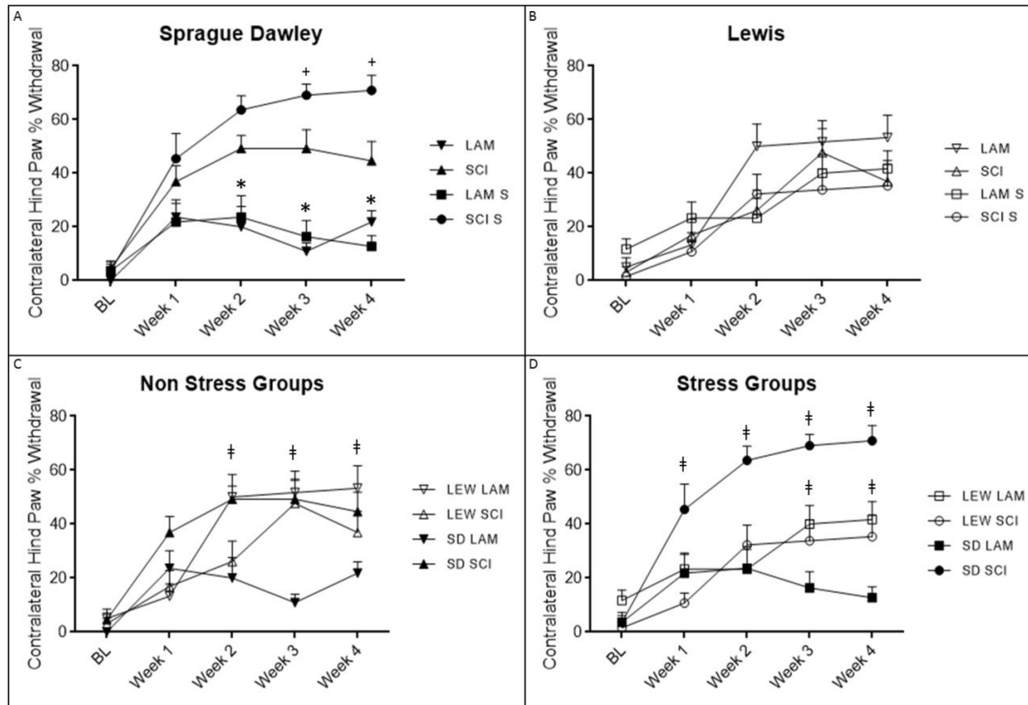


Figure 7: Injury increases cold sensitivity and is affected by strain and stress exposure. SD SCI rats had a greater incidence of paw withdrawal from weeks 1 to 4 compared to their respective LAM groups. SCI animals exposed to stress prior to injury had higher incidence of paw withdrawal compared to non-stressed SCI rats which began at week 3 p..o. and persisted until the end of the study (A). There was significant effect of SCI or stress in LEW rats (B). SD SCI S rats had significantly higher rates of paw withdrawal from weeks 1 to 4 p.o. compared to LEW SCI S rats indicating a strain effect. A strain effect was also observed between SD and LEW LAM S animals. LEW LAM S animals had a higher incidence of paw withdrawal compared to SD rats from weeks 3 to 4 p.o. (C). At week 2 p.o. a strain effect was observed, LEW LAM rats had a greater incidence of paw withdrawal compared to SD LAM animals from weeks 2 to 4 p.o. (D). Data shown are means ± s.e. analyzed via two-way ANOVA with significance set at $p \leq 0.05$. *: significance between SCI and LAM groups. +: significance between S and control

groups. ǂ: significance between SD and LEW groups. SD sample sizes: LAM n = 11, LAM S n = 11, SCI n = 13 and SCI S n = 11. LEW sample sizes: LAM n = 12, LAM S n = 12, SCI n = 13, and SCI S n = 13.

References

1. Johan A. Aarli, T.D., Aleksandar Janca, and Anna Muscetta *Neurological Disorders: public health challenges*. 2006, World Health Organization: Switzerland. p. 218.
2. Wasner, G. and G. Deuschl, *Pains in Parkinson disease--many syndromes under one umbrella.* Nat Rev Neurol, 2012. **8**(5): p. 284-94.
3. Nascimento, P.S.a.O.J.M., *What do general neurologists need to know about neuropathic pain?* Arquivos de Neuro-Psiquiatria, 2009. **67**(3-A).
4. Bennett, A.D., A.W. Everhart, and C.E. Hulsebosch, *Intrathecal administration of an NMDA or a non-NMDA receptor antagonist reduces mechanical but not thermal allodynia in a rodent model of chronic central pain after spinal cord injury.* Brain Res, 2000. **859**(1): p. 72-82.
5. Chu, L.W., et al., *Atorvastatin prevents neuroinflammation in chronic constriction injury rats through nuclear NFkappaB downregulation in the dorsal root ganglion and spinal cord.* ACS Chem Neurosci, 2015. **6**(6): p. 889-98.
6. Costa, B., et al., *AM404, an inhibitor of anandamide uptake, prevents pain behaviour and modulates cytokine and apoptotic pathways in a rat model of neuropathic pain.* Br J Pharmacol, 2006. **148**(7): p. 1022-32.
7. Detloff, M.R., R.E. Wade, Jr., and J.D. Houle, *Chronic at- and below-level pain after moderate unilateral cervical spinal cord contusion in rats.* J Neurotrauma, 2013. **30**(10): p. 884-90.
8. Ellis, A., et al., *Systemic administration of propentofylline, ibudilast, and (+)-naltrexone each reverses mechanical allodynia in a novel rat model of central neuropathic pain.* J Pain, 2014. **15**(4): p. 407-21.
9. Hulsebosch, C.E., et al., *Mechanisms of chronic central neuropathic pain after spinal cord injury.* Brain Res Rev, 2009. **60**(1): p. 202-13.
10. Miyoshi, K., et al., *Interleukin-18-mediated microglia/astrocyte interaction in the spinal cord enhances neuropathic pain processing after nerve injury.* J Neurosci, 2008. **28**(48): p. 12775-87.
11. Wieseler, J., et al., *Unilateral T13 and L1 dorsal root avulsion: methods for a novel model of central neuropathic pain.* Methods Mol Biol, 2012. **851**: p. 171-83.
12. Bardin, L., et al., *Chronic restraint stress induces mechanical and cold allodynia, and enhances inflammatory pain in rat: Relevance to human stress-associated painful pathologies.* Behav Brain Res, 2009. **205**(2): p. 360-6.
13. Kanai, Y., et al., *Involvement of an increased spinal TRPV1 sensitization through its up-regulation in mechanical allodynia of CCI rats.* Neuropharmacology, 2005. **49**(7): p. 977-84.
14. Costa, B., et al., *Vanilloid TRPV1 receptor mediates the antihyperalgesic effect of the nonpsychoactive cannabinoid, cannabidiol, in a rat model of acute inflammation.* Br J Pharmacol, 2004. **143**(2): p. 247-50.
15. Putatunda, R., et al., *Chronic at-level thermal hyperalgesia following rat cervical contusion spinal cord injury is accompanied by neuronal and astrocyte activation and loss of the astrocyte glutamate transporter, GLT1, in superficial dorsal horn.* Brain Res, 2014. **1581**: p. 64-79.
16. Shi, M., et al., *Increased thermal and mechanical nociceptive thresholds in rats with depressive-like behaviors.* Brain Res, 2010. **1353**: p. 225-33.
17. Anke, A.G., A.E. Stenehjem, and J.K. Stanghelle, *Pain and life quality within 2 years of spinal cord injury.* Paraplegia, 1995. **33**(10): p. 555-9.
18. Ataoglu, E., et al., *Effects of chronic pain on quality of life and depression in patients with spinal cord injury.* Spinal Cord, 2013. **51**(1): p. 23-6.
19. Breivik, H., et al., *Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment.* Eur J Pain, 2006. **10**(4): p. 287-333.

20. Gureje, O., et al., *Persistent pain and well-being: a World Health Organization Study in Primary Care.* Jama, 1998. **280**(2): p. 147-51.
21. Hammell, K.R., *Spinal cord injury rehabilitation research: patient priorities, current deficiencies and potential directions.* Disabil Rehabil, 2010. **32**(14): p. 1209-18.
22. Cardenas, D.D., et al., *Gender and minority differences in the pain experience of people with spinal cord injury.* Archives of Physical Medicine and Rehabilitation, 2004. **85**(11): p. 1774-1781.
23. Siddall, P.J., et al., *A longitudinal study of the prevalence and characteristics of pain in the first 5 years following spinal cord injury.* Pain, 2003. **103**(3): p. 249-257.
24. Zimmermann, M., *Pathobiology of neuropathic pain.* European Journal of Pharmacology, 2001. **429**: p. 5.
25. Marchand, F., M. Perretti, and S.B. McMahon, *Role of the immune system in chronic pain.* Nat Rev Neurosci, 2005. **6**(7): p. 521-32.
26. Avishai-Eliner, S., et al., *Altered regulation of gene and protein expression of hypothalamic-pituitary-adrenal axis components in an immature rat model of chronic stress.* J Neuroendocrinol, 2001. **13**(9): p. 799-807.
27. Banu, A.Z.a.N., *Induction of oxidative stress by restraint stress and corticosterone treatments in rats.* Indian Journal of Biochemistry & Biophysics, 2009. **46**: p. 6.
28. Chappell, P.B., et al., *Alterations in corticotropin-releasing factor-like immunoreactivity in discrete rat brain regions after acute and chronic stress.* J Neurosci, 1986. **6**(10): p. 2908-14.
29. Chrousos, G.P., *The role of stress and the hypothalamic–pituitary–adrenal axis in the pathogenesis of the metabolic syndrome: neuro-endocrine and target tissue-related causes.* International Journal of Obesity, 2000. **24**: p. S50-S55.
30. Cullinan, J.P.H.a.W.E., *Neurocircuitry of stress: central control of the hypothalamo–pituitary–adrenocortical axis.* Trends Neurosci, 1997. **20**: p. 7.
31. McEwen, B., *Central effects of stress hormones in health and disease: understanding the protective and damaging effects of stress and stress mediators.* Eur J Pharmacol, 2008. **583**: p. 21.
32. McEwen, B.S., *Protective and damaging effects of stress mediators.* New England Journal of Medicine, 1998. **338**(3): p. 9.
33. Radley, J.J., et al., *Chronic behavioral stress induces apical dendritic reorganization in pyramidal neurons of the medial prefrontal cortex.* Neuroscience, 2004. **125**(1): p. 1-6.
34. Sorrells, S.F., et al., *The stressed CNS: when glucocorticoids aggravate inflammation.* Neuron, 2009. **64**(1): p. 33-9.
35. Bradesi, S., Eutamene, H. , Garcia-Villar, R. , Fioramonti, J. and Bueno, L., *Acute and chronic stress differentially affect visceral sensitivity to rectal distension in female rats.* Neurogastroenterology & Motility, 2002. **14**: p. 8.
36. Robbins, M.T. and T.J. Ness, *Footshock-induced urinary bladder hypersensitivity: role of spinal corticotropin-releasing factor receptors.* J Pain, 2008. **9**(11): p. 991-8.
37. Alexander, J.K., et al., *Stress exacerbates neuropathic pain via glucocorticoid and NMDA receptor activation.* Brain Behav Immun, 2009. **23**(6): p. 851-60.
38. Bravo, L., Mico, J.A., Rey-Brea, R., Perez-Nievas, B., Leza, J.C., Berrocoso, E., *Depressive-like states heighten the aversion to painful stimuli in a rat model of comorbid chronic pain and depression.* Anesthesiology, 2012. **117**(3): p. 613-625.
39. Bravo, L., et al., *Social stress exacerbates the aversion to painful experiences in rats exposed to chronic pain: the role of the locus coeruleus.* Pain, 2013. **154**(10): p. 2014-23.
40. Lidia Bravo, J.A.M., Raquel Rey-Brea, A.S., Beatriz Perez-Nievas, Juan Carlos Leza, Esther Berrocoso, *Depressive-like States Heighten the Aversion to Painful Stimuli in a Rat Model of Comorbid Chronic Pain and Depression.* Anesthesiology, 2012. **117**: p. 13.
41. Bradesi, S., et al., *Role of spinal microglia in visceral hyperalgesia and NK1R up-regulation in a rat model of chronic stress.* Gastroenterology, 2009. **136**(4): p. 1339-48, e1-2.

42. Zafir, A. and N. Banu, *Modulation of in vivo oxidative status by exogenous corticosterone and restraint stress in rats.* Stress, 2009. **12**(2): p. 167-77.
43. Hausmann, O.N., *Post-traumatic inflammation following spinal cord injury.* Spinal Cord, 2003. **41**(7): p. 369-78.
44. Rafati, D.S., et al., *Nuclear factor-kappaB decoy amelioration of spinal cord injury-induced inflammation and behavior outcomes.* J Neurosci Res, 2008. **86**(3): p. 566-80.
45. Bethea, J.R., et al., *Traumatic spinal cord injury induces nuclear factor-kappa B activation.* Journal of Neuroscience, 1998. **18**(9): p. 3251-3260.
46. KEANE, R.W., KRAYDIEH, S., LOTOCKI, G., BETHEA, J.R., KRAJEWSKI, S., REED, J.C., AND DIETRICH, W.D., *Apoptotic and anti-apoptotic mechanisms following spinal cord injury.* J Neuropathol Exp Neuro, 2001. **60**(5): p. 8.
47. Oyinbo, C.A., *Secondary injury mechanisms in traumatic spinal cord injury:a nugget of this multiply cascade.* Acta Neurobiologiae Experimentalis, 2011. **71**: p. 19.
48. Eugene Park Alexander A. Velumian, a.M.G.F., *The Role of Excitotoxicity in Secondary Mechanisms of Spinal Cord Injury: A Review with an Emphasis on the Implications for White Matter Degeneration.* Journal of Neurotrauma, 2004. **21**(6): p. 21.
49. Charles H. tator, M.G.F., *Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms.* J Neurosurg, 1991. **75**: p. 12.
50. Black, L.V., T.J. Ness, and M.T. Robbins, *Effects of oxytocin and prolactin on stress-induced bladder hypersensitivity in female rats.* J Pain, 2009. **10**(10): p. 1065-72.
51. Dunham, K.A., et al., *Characterization of a graded cervical hemicontusion spinal cord injury model in adult male rats.* J Neurotrauma, 2010. **27**(11): p. 2091-106.
52. Mestre, H., et al., *Lewis, Fischer 344, and sprague-dawley rats display differences in lipid peroxidation, motor recovery, and rubrospinal tract preservation after spinal cord injury.* Front Neurol, 2015. **6**: p. 108.
53. Charles D. Mills, B.C.H., Kathia M. Johnson, and Claire E. Hulsebosch, *Strain and Model Differences in Behavioral Outcomes after Spinal Cord Injury in Rat.* J Neurotrauma, 2001. **18**: p. 14.
54. Schmitt, C., et al., *Changes in spinal cord injury-induced gene expression in rat are strain-dependent.* Spine J, 2006. **6**(2): p. 113-9.
55. Chaovipoch, P., et al., *17beta-estradiol is protective in spinal cord injury in post- and pre-menopausal rats.* J Neurotrauma, 2006. **23**(6): p. 830-52.
56. Kachadroka, S., et al., *Effect of endogenous androgens on 17beta-estradiol-mediated protection after spinal cord injury in male rats.* J Neurotrauma, 2010. **27**(3): p. 611-26.
57. Siriphorn, A., S. Chompoopong, and C.L. Floyd, *17beta-estradiol protects Schwann cells against H2O2-induced cytotoxicity and increases transplanted Schwann cell survival in a cervical hemicontusion spinal cord injury model.* J Neurochem, 2010. **115**(4): p. 864-72.
58. Sribnick, E.A., et al., *Estrogen attenuated markers of inflammation and decreased lesion volume in acute spinal cord injury in rats.* J Neurosci Res, 2005. **82**(2): p. 283-93.
59. Yune, T.Y., et al., *Systemic administration of 17beta-estradiol reduces apoptotic cell death and improves functional recovery following traumatic spinal cord injury in rats.* J Neurotrauma, 2004. **21**(3): p. 293-306.
60. Hauben, E., et al., *Sexual dimorphism in the spontaneous recovery from spinal cord injury: a gender gap in beneficial autoimmunity?* Eur J Neurosci, 2002. **16**(9): p. 1731-40.
61. Center, T.N.S.S., *Spinal Cord Injury Fact and Figures at a Glance*, U.o.A.a. Birmingham, Editor. 2013, University of Alabama at Birmingham: Birmingham, AL.
62. Center, T.N.S.S., *Spinal Cord Injury Fact and Figures at a Glance*, U.o.A.a. Birmingham, Editor. 2016, University of Alabama at Birmingham: Birmingham, AL.
63. Tache, Y. and B. Bonaz, *Corticotropin-releasing factor receptors and stress-related alterations of gut motor function.* J Clin Invest, 2007. **117**(1): p. 33-40.

64. Rich, E.L. and L.M. Romero, *Exposure to chronic stress downregulates corticosterone responses to acute stressors.* Am J Physiol Regul Integr Comp Physiol, 2005. **288**(6): p. R1628-36.
65. Hauger, R.L., et al., *CRF receptor regulation and sensitization of ACTH responses to acute ether stress during chronic intermittent immobilization stress.* Brain Res, 1990. **532**(1-2): p. 34-40.
66. Burchfield, S.R., S.C. Woods, and M.S. Elich, *Pituitary adrenocortical response to chronic intermittent stress.* Physiol Behav, 1980. **24**(2): p. 297-302.
67. Anna M. Aloisi, H.L.S., Nanne E. Van De Polli and Francesca Farabollini, *Sex-Dependent Effects of Restraint on Nociception and Pituitary-Adrenal Hormones in the Rat.* Physiol Behav, 1994. **55**: p. 5.
68. Babb, J.A., Masini, C. V., Day, H. E. and Campeau, S., *Sex differences in activated corticotropin-releasing factor neurons within stress-related neurocircuitry and hypothalamic-pituitary-adrenocortical axis hormones following restraint in rats.* Neuroscience, 2013. **234**: p. 40-52.
69. Glenn T. Livezey, J.M.M.a.W.H.V., *Plasma norepinephrine, epinephrine and corticiosterone stress responses to restraint in individual male and female rats, and their correlations.* Neurosci Lett, 1985. **62**: p. 6.
70. G. Jean Kant, R.H.L., Bradford N. Bunnell, Edward H. Mougey, Lee l. Pennington and James l. Meyerhoff, *Comparison of stress response in male and female rats: Pituitary cyclic AMP and plasma prolactin, growth hormone, and corticosterone.* PSYCHONEUROENDOCRINO, 1983. **8**: p. 8.
71. Goel, N., et al., *Sex differences in the HPA axis.* Compr Physiol, 2014. **4**(3): p. 1121-55.
72. Robbins, M.T., et al., *Footshock stress differentially affects responses of two subpopulations of spinal dorsal horn neurons to urinary bladder distension in rats.* Brain Res, 2011. **1386**: p. 118-26.
73. van Gorp, S., et al., *Translation of the rat thoracic contusion model; part 1-supraspinally versus spinally mediated pain-like responses and spasticity.* Spinal Cord, 2014. **52**(7): p. 524-8.

POTENTIAL THERAPEUTIC EFFICACY OF CANNABIDIOL ON SECONDARY INJURY POST SPINAL CORD INJURY

by

AMANDA MOHAIMANY-APONTE, SEAN D. MCALLISTER, AND CANDACE L. FLOYD

In preparation for *Journal of Neurotrauma*

Format adapted for dissertation

Potential therapeutic efficacy of cannabidiol on secondary injury post spinal cord injury

Amanda Mohaimany-Aponte[1], Sean D. McAllister, Ph.D.[2], and Candace L. Floyd, Ph.D.[1]

[1]University of Alabama at Birmingham, Birmingham, AL and [2]California Pacific Medical Center Research Institute, San Francisco, CA

Abstract

The following review is a discussion on the potential therapeutic benefit of cannabidiol (CBD) for spinal cord injury (SCI), specifically in regards to secondary injury and SCI induced neuropathic pain (SCI-NP). Within the SCI population, SCI-NP development affects up to 80% of patients with secondary injury being a major contributor to development and maintenance of SCI-NP. There are few therapeutic options for SCI-NP patients, which negatively impacts quality of life. The literature has shown targeting aspects of this cascade improves outcomes post SCI including ameliorating SCI-NP development. CBD has been shown to target many aspects of secondary injury and penetrate the central nervous system (CNS) within minutes of administration with little to no negative effects. Though CBD has had clinical efficacy in treating NP, it has not been examined in SCI-NP leaving a gap in knowledge of the therapeutic benefits of CBD. This review will discuss how CBD interacts within the CNS, potential mechanisms of action regarding secondary injury, and the therapeutic potential of CBD for imparting protection against SCI-NP development.

Overview

Over 300,000 individuals in the United States are living with chronic spinal cord injury (SCI) and within this year over 17,000 individuals will be added to this staggering number [1]. Nearly 80% of these individuals will go on to develop neuropathic pain induced by SCI (SCI-NP) [2, 3]. SCI-NP greatly diminishes quality of life, and has been shown to increase incidence of depression and anxiety within this patient population due to the lack of effective therapeutic options to treat and manage pain events [4-6]. This devastating issue is frequently listed as a top priority for SCI patients, often being listed above functional recovery [7, 8].

A promising therapeutic that has been evaluated clinically to manage pain stemming from multiple sclerosis and chemotherapy is cannabidiol (CBD) [9, 10]. CBD is a non-psychoactive component of *Cannabis sativa* (*C. sativa*) that has been shown to have anti-oxidant and anti-inflammatory properties, and also has the ability to cross into the central nervous system (CNS) [11-22]. Previous studies have shown that CBD is pleiotropic and can target many different pathways [23-26]. The importance of pursuing a compound that targets many aspects of inflammation and oxidation is that acutely, post mechanical injury to the spinal cord, multiple adverse events occur. These include a rapid and large increase in inflammation, generation of reactive oxygen species (ROS), dysregulation of glutamate and calcium, and immune cell activation. These events termed secondary injury, further increases neuronal damage (Figure 1) [27-31]. Secondary injury can persist for months post SCI and further increases damage to the spinal cord [29]. Studies have shown that targeting secondary injury decrease incidence of SCI-NP [32-35].

CBD has been shown to decrease generation of inflammatory products, ROS, and immune cell activation, leading to enhanced neuroprotection in animal models of inflammation, nerve injury, and autoimmune disorders. CBD however has not been evaluated in treating SCI-NP, leaving a large gap in knowledge. Since CBD has been shown to target many pathways and components that play a role in secondary injury, it is an ideal candidate for investigation in SCI-NP [16, 19, 36, 37]. The ability for CBD to target multiple aspects of secondary injury in addition to its ability to penetrate the CNS with low adverse effects or toxicity makes CBD a promising therapeutic. The purpose of this review is to discuss the therapeutic potential of CBD in the context of secondary injury post SCI.

CBD: Pharmacodynamics, Pharmacokinetics, and the Endocannabinoid System

Unlike other components of *C. sativa*, CBD does not bind efficiently to cannabinoid receptors and has been shown to have some antagonistic activity on these receptors [24, 26, 38]. CBD is pleiotropic, exerting its effects on a multitude of pathways [15, 17-19, 24, 36, 38-41]. Studies investigating the effects of CBD have shown a wide array of outcomes ranging from anti-epileptic, analgesic, anti-inflammatory, anti-oxidant, and anti-anxiety [14-17, 19-21, 23]. In addition to this, CBD has been shown to have low toxicity and few adverse effects in animal models of pain and inflammatory disorders, as well as humans [10, 14, 25].

CBD has the ability to be administered in a variety of ways including oral, subcutaneous, intravenous (I.V.), and inhalation [9, 15, 19, 23, 42, 43]. Depending on route of

administration, CBD presence in the plasma ranges from 5 – 30 minutes with I.V. administration being the quickest route and oral being the slowest [11, 26, 43]. Bioavailability of CBD in humans has shown variability between individuals, ranging from 11% – 45 with an average half-life of 24 hours (ranging from 18 – 33 hours between patients) after inhalation or I.V. administration of drug [26, 44] [45-48]. CBD is subject to first pass effects after oral administration, meaning it undergoes hepatic metabolism prior to reaching systemic circulation [49-51]. Studies investigating the pharmacokinetics of CBD and additional cannabinoids in mice and rats demonstrate penetrance and rapid distribution to the brain [11, 52, 53].

Many *C. sativa* derived compounds produce their effects through cannabinoid (CB) receptors, CB_1 and CB_2, which are a component of the endocannabinoid system [54, 55]. CB_1 and CB_2 are G protein-coupled receptors (GPCR) present in the CNS as well as in the peripheral nervous system (PNS), with CB_1 being more highly expressed in the CNS compared to CB_2 which is more greatly expressed on microglia and other immune cells [56, 57]. The endocannabinoid system consists of CB receptors and endogenous ligands, termed endocannabinoids, which are synthesized, removed, and degraded through specific pathways [58]. This system is involved in many processes including memory, immune function, appetite, sleep, and pain. Endocannabinoids are lipophilic and are derived from arachidonic acid [33, 55, 59]. Ligand binding to CB_1 and CB_2 leads to inhibition of neurotransmitter release, thereby causing decreasing neuronal activity. The binding of Δ^9-THC to these receptors cause the psychotropic effects associated *C. sativa* use. Activation of the CB receptors leads to modulation of neuronal and immune cell activity through GPCR activation of specific G-proteins. CB_1 has been shown to act upon

both G_o and G_i G-proteins depending on which ligand binds to CB_1 [54]. G_o is highly expressed in the CNS and has been shown to inhibit calcium channels, whereas G_i has been shown to inhibit adenylate cyclase activity, and activate potassium channel activity [60-62]. These inhibitory and stimulatory effects of CB_1 lead to decreased release of neurotransmitters such as glutamate and gamma-Aminobutyric acid (GABA), again depending on which ligand binds to the receptor [63, 64]. CB_2 has been shown to act upon G_o, and activation of this G-protein has been shown to decrease microglia activity [65]. CBD does not activate the CB receptors, and has shown slight antagonistic activity on these receptors [66]. CBD also structurally acts as an antioxidant, specifically by interacting with and neutralizing oxidative products and by donating electrons [13].

In addition to the CB receptors, transient receptor potential vanilloid receptor (TRPV1) has been shown to be a target receptor for cannabinoids and endocannabinoids, and has been termed the endovanilloid system [67]. TRPV1 is cation channel that is activated upon ligand binding, is expressed on neurons, and activation of has been shown to increase influx of calcium into the cell, thereby increasing neuronal activity [68]. In regards to neuropathic pain, TRPV1 activity has been shown to enhance pain and that blocking TRPV1 diminishes pain [69-71]. A study investigating the therapeutic efficacy of CBD in ameliorating a hyperalgesic pain phenotype and the receptor selectivity of CBD on the endocannabinoid and endovanilloid system found that administration of CBD decreased incidence of thermal hypersensitivity in an animal model of inflammation, and that CBD exerted these effects by acting as a TRPV1 antagonist [72]. Beyond this, a study investigating liver inflammation, found that CBD decreased inflammation, however, reduced effects where observed in TRPV1 knockout animals

[73]. These studies show that CBD in part exerts effects through antagonism of TRPV1. It is important to note that CBD is pleiotropic and exerts a multitude of effects through different biological pathways, which will be discussed in this review.

CBD: Modulation of Secondary Injury Pathways

Glutamate Mediated Excitotoxicity

Acutely post SCI, glutamate is released extracellularly which causes increased excitatory events, in addition to excessive depolarization and neuronal activity, exposure to high levels of glutamate can induce cell death; which is mediated by several sources post injury such as necrotic and lysed neuronal cells, release from presynaptic neurons, and dysfunction in glutamate receptors [74-76]. CBD has been shown to antagonize N-methyl-D-aspartate (NMDA) and 2-amino-3-(4-butyl-3-hydroxylsoxazol-5-yl) propionic acid (AMPA)/kainite receptors, which contribute to glutamate excitotoxicity [8, 77-83]. The ability of CBD to antagonize NMDA and AMPA is linked to efficacy in reducing seizures and epileptic events in rodents. CBD has also recently garnered much attention for its potential therapeutic use in children and young adults with treatment-resistant epilepsy [84-86]. A study investigating the effects of Δ^9-THC and CBD on NMDA induced toxicity in the retina found that both Δ^9-THC and CBD diminished neurotoxicity mediated by NMDA. Specifically, it was determined that high activity of NMDA increased production of oxidative products and that CBD treatment reduced the concentration of these oxidative species as well as apoptosis induced by NMDA [78]. The importance of examining the effects of glutamate mediated toxicity and the receptors

involved is that when glutamate binds to these receptors, calcium is able to enter the cell. The influx of calcium can induce cell damage and death, thereby increasing cellular damage post SCI.

A study looking at concentrations of glutamate found that sex affects glutamate concentrations in the rat brain, and that the stage of estrous affects glutamate concentration [87]. In addition to this, a study investigating the effects of female sex hormones on the modulation of behavioral conditions in rodents determined that estrogen affects synaptic plasticity in the brain by increasing dendritic spine length and decreased glutamate receptor expression [88]. In addition to differences in glutamate concentration, receptor expression, and synaptic plasticity differences between male and female animals, a study examining behavioral effects of an NMDA antagonist in male and female rodents found that female rats had greater susceptibility to NMDA receptor antagonists compared to male rodents [89]. Currently, no study has examined the action of CBD on glutamate mediated excitotoxicity post SCI in male and female rodents; therefore, no clear insight can be made on how efficacious CBD will be at targeting this aspect of secondary injury.

Intracellular Calcium Release

After SCI, glutamate is released which causes NMDA channels to open, which then allows calcium to enter the cell [74-76, 90, 91]. A study investigating the role of calcium-activated neutral proteinase (calpain) in secondary injury post SCI found that calpain expression and activity was increased post SCI within the primary injury site, as well as regions rostral and caudal to injury acutely after SCI [92]. Calpain, an enzyme activated by calcium, has been shown to have a role in inducing apoptosis in the CNS and that

blocking calpain activity post SCI reduced apoptosis, COX-2 activity, and improved motor function [93-95]. In addition to increasing enzymatic activity, intracellular calcium influx has been shown to damage mitochondria, which have been shown to act as an intracellular calcium buffer by taking up calcium. The damage to mitochondria has led to reduced cellular respiration, increased generation of ROS, and cell death [96-98]. Cannabinoids have been shown to modulate the activity mitochondria, specifically; in the brain, heart, and pulmonary cells, Δ^9-THC has been shown to interact with the mitochondrial electron transport chain leading to a decrease oxidase activity [99, 100]. In regards to calcium uptake, a study investigating calcium uptake by synaptosomes in the brain and the modulatory effects of cannabinoids on uptake found that Δ^9-THC blocked the ability of synaptosomes to take up calcium post depolarization, whereas CBD had no ability to block calcium depolarization-dependent uptake but was able to block calcium transport [101]. Several studies have found that CBD administration in non-excitotoxic states has led to an increase in intracellular calcium in a variety of cell types including hippocampal cells [102-104]. A study examining calcium regulation by CBD found that under hyper-excitability, high potassium conditions, CBD reduced intracellular calcium concentrations in hippocampal cells [103]. In addition to these findings, it was determined that CBD was exerting these effects through the mitochondria, specifically the sodium calcium exchanger [103]. These studies indicate that during secondary injury, CBD can reduce intracellular calcium levels and have neuroprotective effects.

Immune Cell Activity and Inflammatory Product Generation

The resident immune cell of the CNS, microglia, are activated acutely after SCI, with pro-inflammatory phenotype peaking approximately seven days post injury and reaching

fifty percent peak concentration around 55 days post SCI. This indicates microglia and macrophages are chronically present at the site of injury, and additionally have been shown to contribute to pain post SCI [105-108]. Beyond microglia, other immune cells, such as neutrophils and T cells are recruited to the site of injury, with infiltration occurring within one week of injury [29, 108]. CBD has been shown to diminish the concentration of pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6, which are produced by immune cells, primarily macrophages which are chronically present in the tissue post SCI [15, 18, 19, 21, 109]. A study investigating the effects of CBD on diabetic cardiomyopathy found that CBD decreased caspase 3/7 activity, an indicator of mitochondrial induced apoptosis, as well as 1,3-nitrotyrosine (3-NT) which is a marker of oxidative stress mediated by mitochondrial damage; overall indicating that CBD may be reducing mitochondrial damage during events of cellular stress, thereby diminishing generation of inflammatory and oxidative products [20].

Serotonergic Pathway

SCI results in loss and damage to several neuronal pathways, one being the serotonergic pathway. A study investigating serotonin concentrations in the spinal cord post injury found that the larger the loss of serotonergic neurons after SCI, the greater the severity of injury and loss of motor function [110]. Other studies have found that serotonergic neurons contribute to preservation and recovery of motor function post SCI, and that the type of serotonin receptor being expressed can cause different effects of locomotion [111-113]. Beyond motor function, the serotonergic system has been shown to modulate pain in the spinal cord. A study examining the effects of administration of serotonin directly to the spinal cord on thermal nociception found that administration of serotonin reduced

nociception to thermal stimuli in three animal models (rat, rabbit, and cat) [114]. A study investigating the effects of serotonin on morphine mediated analgesia found that blocking serotonergic receptors decreased analgesic effects and that administration of serotonin with morphine enhanced analgesic effects, indicating that activation of serotonergic pathways can relieve pain [115]. CBD has been shown to have agonistic activity at the serotonin receptor 5-hydroxytryptamine, 5-HT subtype 1A ($5\text{-}HT_{1A}$) [116]. A study investigating the efficacy of CBD on treating chemotherapy-induced neuropathy in mice found that CBD reduced mechanical allodynia in a dose dependent manner and that CBD treatment did not result in addiction-like behaviors [117]. In addition to this, it was found that blocking 5-HT receptors diminished the effectiveness of CBD treatment whereas blocking CB receptors did not affect CBD effectiveness. These results indicate that CBD has the ability to ameliorate neuropathic pain, and exerts these effects in part by acting upon serotonergic receptors. The $5\text{-}HT_{1A}$ receptor is coupled to a GPCR G_i complex, which leads to inhibition of adenylate cyclase activity and causes increased activity of potassium channels which causes hyperpolarization of the cell [61]. A study investigating SCI hemisection at the thoracic vertebral level 13 found that injury induced changes to serotonin, 5-HT transporter, and pain in a rodent model found that post injury there was a decrease in serotonergic neurons within the dorsal laminae of the lumbar region of the spinal cord but not the cervical region as well as an increase in 5-HT transporter expression below lesion, indicating below lesion loss of the serotonergic pathway and perhaps a compensatory mechanism by surviving serotonergic neurons. In addition to this, it was found that below lesion there were decreased concentrations of serotonin ipsilateral to injury.

Beyond this, it was determined that there was no development of allodynic or hyperalgesic pain above lesion, but there was development of both allodynic and hyperalgesic pain below level of the lesion, in addition , it was determined that exogenous administration of serotonin diminished allodynic pain in a dose dependent manner, and attenuated hyperalgesic pain at the highest dose, and that administration of serotonin receptor blocker negated these effects and that blocking serotonin reuptake had little to no effect on ameliorating pain [118]. It should be noted that previous studies show an increase in plasma concentrations of serotonin post SCI, however these studies were conducted acutely post injury whereas the study discussed looked at a chronic time point post SCI (28 days) [119, 120]. Considering this, it is possible that CBD could activate serotonergic receptors during a time where serotonin is reduced at the site of injury, contributing to diminished mitochondrial damage, thereby inducing neuroprotective effects and ameliorating pain development post SCI.

Peroxisome proliferator-activated receptors (PPAR)

PPAR regulate transcriptional activity via blocking nuclear translocation or blocking DNA binding sites [121-123]. There are three isoforms of PPAR, PPARα, PPARβ, and PPARγ, with PPARγ being highly expressed in the CNS compared to PPARα and PPARβ [122, 124, 125]. Beyond expression in the CNS, PPARγ is also expressed in a wide variety of cell types such as adipose, liver, immune and gastrointestinal [40, 126-132]. There is a large amount of evidence indicating PPAR downregulates activity of nuclear factor-kappa b (NF-κB) [122, 123, 133-137]. NF-κB, similar to PPAR, modulates transcriptional activity [138]. In the context of SCI, NF-κB activation leads to increased transcription of pro-inflammatory compounds [27, 31, 139, 140]. Previous studies have

shown that increasing PPAR activity decreases NF-κB activity, indicating a potential way to target secondary injury post SCI and therefore stop the spread of neuronal damage out from the site of injury [133, 137]. CBD has been shown to increase activation of PPARγ [16, 17, 141, 142]. A study investigating CBD modulation of intestinal inflammation via the neuroimmune axis found that blocking PPARγ receptors diminished the ability of CBD to reduce inflammation in the intestine and that blocking CBD action on PPARγ increased the concentration of glial cells compared [16].

Conclusion

Secondary injury resulting from SCI has been shown to increase and spread damage rostral and caudal from the primary injury site [29, 30]. In addition to this, it has been shown to last chronically in SCI patients which further increases damage to the spinal cord [29]. Currently, there is no standardized treatment for secondary injury and the main focus of acute care post SCI is stabilizing the patient, assessing their neurological and functional state, and potentially decompressing the spinal cord through surgical intervention [143-146]. Major issues facing treatment for SCI and secondary injury is ability for the treatment to penetrate the CNS, target secondary injury with few adverse effects, and patient variability as the major cause of SCI is due to vehicular accident and falls and not disease state [1, 7, 8]. These issues have created an inability to treat secondary injury in the human SCI population and have led to a variety of chronic disorders that severely diminish the quality of life for these patients. A chronic disorder that a vast majority of SCI patients, nearly 80%, go on to develop is neuropathic pain.

SCI-NP is frequently listed as a top concern for SCI patients as there are few effective treatments to prevent onset of pain or manage pain events when they occur. SCI-NP has been shown to decrease quality of life greatly through increased incidence of anxiety, depression, sleep loss, and decreased work productivity. Another compounding factor is that there is a large male skew in the SCI population and majority of chronic pain disorders affect females [1, 4-6]. The skew is not due to epidemiological factors, as SCI is commonly caused by accidents and falls, but primarily due to sex differences as estrogen and estrogen-related hormones have been shown to be protective in animal models of SCI [1, 147-152]. Estrogen has been shown to be protective in secondary injury post SCI, and as discussed in this review, has been shown to be protective in a majority of secondary injury factors such as regulating intracellular calcium concentrations and PPARγ concentrations in the CNS [87-89, 126, 128, 132, 149-158]. Studies have shown that reducing aspects of secondary injury, i.e. inflammation, immune cell activation, glutamate, reduces incidence of neuropathic pain in a variety of injury models, including SCI [32, 33, 35, 69, 70, 159, 160]. These studies, though having advanced knowledge of secondary injury's contribution to neuropathic pain development and maintenance, have approached these investigations by addressing secondary injury via one characteristic. As previous studies have shown, secondary injury is multifactorial and many of the factors feed into one another [27-30, 161, 162]. For example, primary injury induces release of glutamate which leads to excitotoxicity and the activation of ion channels that lead to high concentrations of intracellular calcium that in turn increase production of ROS and mitochondrial damage [74-76, 90, 96-98]. Therefore, addressing one aspect of secondary injury may not be enough to diminish SCI-NP development.

CBD can penetrate the CNS with few adverse effects and low toxicity, and has been shown to beneficially target multiple aspects of secondary injury in a variety of disease models [11, 14, 23-25, 38, 39, 49, 52, 163]. Beyond animal models, CBD has shown clinical efficacy in ameliorating neuropathic pain in a variety of disorders [9, 10, 79]. A clinical trial using a cannabinoid formulation of 1:1 Δ^9-THC to CBD (Sativex) in patients with chronic SCI to manage spasticity and neuropathic pain, found that patients who were given the drug reported increased quality of life and decreased pain scores, but that the drug treatment did not decrease the number of spastic events nor pain episodes [164]. It should be noted that using Δ^9-THC for treatment still has the potential to elicit unwanted psychotropic effects, whereas CBD has been shown to be non-psychotropic and reduce pain. Therefore, examining the efficacy of CBD treatment alone in this patient population is of great interest.

Current clinical studies with cannabinoids aim to manage neuropathic pain that has already developed. This is an important aspect to consider for SCI patients living with chronic SCI, estimated to be over 300,000 individuals in the United States and over 2.5 million worldwide [1, 4]. However, it is also import to consider using CBD as a therapeutic to prevent development of SCI-NP in order to treat the over 17,000 annual cases of SCI in the United States and over 500,000 worldwide [1, 4]. Since SCI can not be prevented due to the accidental nature of its onset, targeting secondary injury is the key to treating SCI. As previous studies have shown, CBD is a highly suitable drug candidate for treating secondary injury post SCI by modulating multiple aspects of this injury cascade such as immune cell activation, generation of inflammatory and oxidative species, and glutamate mediated excitotoxicity. Considering this, it must also be noted

that there is no one factor of secondary injury solely responsible for the spread of damage post SCI or development of SCI-NP. As this review has discussed, multiple studies have shown many different components contribute to these events. Taken together, the inevitable induction and multifactorial nature of secondary injury, the drug candidate of choice must be able to be easily administered, reach the CNS quickly, and target secondary injury with little to no adverse effects. As this review has discussed, and the literature has shown, CBD may be a promising therapeutic for the treatment of secondary injury post SCI.

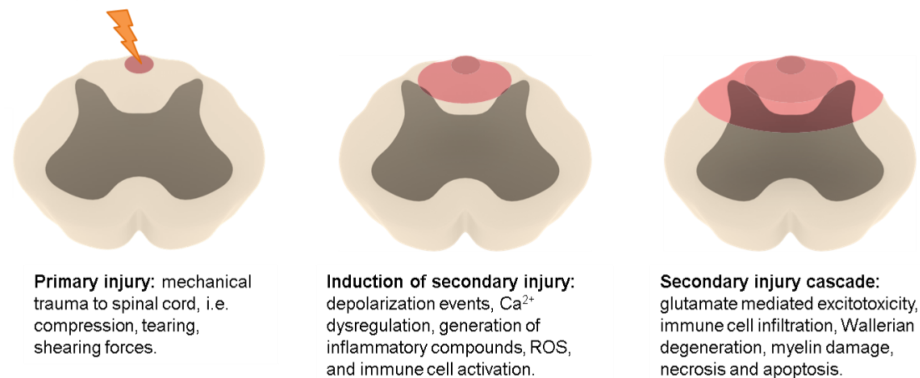


Figure 1: Secondary injury spreads damage. Primary injury, mechanical damage to the spinal cord, induces a secondary injury cascade. Secondary injury is characterized by glutamate induced excitotoxicity, calcium dysregulation, generation of reactive species and inflammatory products, and necrosis. These factors damage surrounding neurons, and spread neuronal damage. In addition to increased cellular damage, the secondary injury cascade increases inflammation which perpetuates the secondary injury cascade. Secondary injury has been shown to last chronically, up to a year after SCI in humans.

References:

1. Center, T.N.S.S., *Spinal Cord Injury Fact and Figures at a Glance*, U.o.A.a. Birmingham, Editor. 2016, University of Alabama at Birmingham: Birmingham, AL.
2. Breivik, H., et al., *Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment.* Eur J Pain, 2006. 10(4): p. 287-333.
3. Gureje, O., et al., *Persistent pain and well-being: a World Health Organization Study in Primary Care.* Jama, 1998. 280(2): p. 147-51.
4. Johan A. Aarli, T.D., Aleksandar Janca, and Anna Muscetta *Neurological Disorders: public health challenges*. 2006, World Health Organization: Switzerland. p. 218.
5. Bouhassira, D., et al., *Comparison of pain syndromes associated with nervous or somatic lesions and development of a new neuropathic pain diagnostic questionnaire (DN4).* Pain, 2005. 114(1-2): p. 29-36.
6. Bouhassira, D., et al., *Prevalence of chronic pain with neuropathic characteristics in the general population.* Pain, 2008. 136(3): p. 380-7.
7. Anke, A.G., A.E. Stenehjem, and J.K. Stanghelle, *Pain and life quality within 2 years of spinal cord injury.* Paraplegia, 1995. 33(10): p. 555-9.
8. Siddall, P.J., et al., *A longitudinal study of the prevalence and characteristics of pain in the first 5 years following spinal cord injury.* Pain, 2003. 103(3): p. 249-257.
9. Rog, D.J., T.J. Nurmikko, and C.A. Young, *Oromucosal delta9-tetrahydrocannabinol/cannabidiol for neuropathic pain associated with multiple sclerosis: an uncontrolled, open-label, 2-year extension trial.* Clin Ther, 2007. 29(9): p. 2068-79.
10. Johnson, J.R., et al., *Multicenter, double-blind, randomized, placebo-controlled, parallel-group study of the efficacy, safety, and tolerability of THC:CBD extract and THC extract in patients with intractable cancer-related pain.* J Pain Symptom Manage, 2010. 39(2): p. 167-79.
11. Alozie, S.O., et al., *3H-delta 9-Tetrahydrocannabinol, 3H-cannabinol and 3H-cannabidiol: penetration and regional distribution in rat brain.* Pharmacol Biochem Behav, 1980. 12(2): p. 217-21.
12. Dewey, W.L., et al., *Distribution of radioactivity in brain of tolerant and nontolerant pigeons treated with 3 H- 9 -tetrahydrocannabinol.* Biochem Pharmacol, 1973. 22(3): p. 399-405.
13. A. J. HAMPSON, M.G., J. AXELROD, AND D.WINK, *Cannabidiol and (-)delta9-tetrahydrocannabinol are neuroprotective antioxidants.* Proc Natl Acad Sci U S A, 1998. 95: p. 6.
14. A. W. Zuardi, I.S., E. Finkelfarb, and I. G. Karniol, *Action of cannabidiol on the anxiety and other effects produced by delta THC in normal subjects.* Psychopharmacology, 1982. 76: p. 6.
15. Costa, B., et al., *The non-psychoactive cannabis constituent cannabidiol is an orally effective therapeutic agent in rat chronic inflammatory and neuropathic pain.* Eur J Pharmacol, 2007. 556(1-3): p. 75-83.
16. De Filippis, D., et al., *Cannabidiol reduces intestinal inflammation through the control of neuroimmune axis.* PLoS One, 2011. 6(12): p. e28159.

17. Esposito, G., et al., *Cannabidiol reduces Abeta-induced neuroinflammation and promotes hippocampal neurogenesis through PPARgamma involvement.* PLoS One, 2011. 6(12): p. e28668.
18. Kozela, E., et al., *Cannabinoids Delta(9)-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-kappaB and interferon-beta/STAT proinflammatory pathways in BV-2 microglial cells.* J Biol Chem, 2010. 285(3): p. 1616-26.
19. Malfait, A.M., et al., *The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis.* Proc Natl Acad Sci U S A, 2000. 97(17): p. 9561-6.
20. Rajesh, M., et al., *Cannabidiol attenuates cardiac dysfunction, oxidative stress, fibrosis, and inflammatory and cell death signaling pathways in diabetic cardiomyopathy.* J Am Coll Cardiol, 2010. 56(25): p. 2115-25.
21. Ribeiro, A., et al., *Cannabidiol improves lung function and inflammation in mice submitted to LPS-induced acute lung injury.* Immunopharmacol Immunotoxicol, 2015. 37(1): p. 35-41.
22. Borrelli, F., et al., *Cannabidiol, a safe and non-psychotropic ingredient of the marijuana plant Cannabis sativa, is protective in a murine model of colitis.* J Mol Med (Berl), 2009. 87(11): p. 1111-21.
23. Bhattacharyya, S., et al., *Opposite effects of delta-9-tetrahydrocannabinol and cannabidiol on human brain function and psychopathology.* Neuropsychopharmacology, 2010. 35(3): p. 764-74.
24. Francois Petitet, B.J., Michel Reibaud, Assunta Imperato and Marie-Christine Dubroeucq, *COMPLEX PHARMACOLOGY OF NATURAL CANNABINOIDS : EVIDENCE FOR PARTIAL AGONIST ACTIVITY OF A'-TETRAHYDROCANNABINOL AND ANTAGONIST ACTIVITY OF CANNABIDIOL ON RAT BRAIN CANNABINOID RECEPTORS.* Life Sciences, 1998. 63(1): p. 6.
25. Zuardi, A.W., *Cannabidiol: from an inactive cannabinoid to a drug with wide spectrum of action.* Revista Brasileira de Psiquiatria, 2008. 30(3): p. 10.
26. Grotenhermen, F., *Pharmacokinetics and Pharmacodynamics of Cannabinoids.* Clin Pharmacokinet, 2003. 42(4): p. 34.
27. Bethea, J.R., et al., *Traumatic spinal cord injury induces nuclear factor-kappa B activation.* Journal of Neuroscience, 1998. 18(9): p. 3251-3260.
28. EUGENE PARK ALEXANDER A. VELUMIAN, a.M.G.F., *The Role of Excitotoxicity in Secondary Mechanisms of Spinal Cord Injury: A Review with an Emphasis on the Implications for White Matter Degeneration.* Journal of Neurotrauma, 2004. 21(6): p. 21.
29. Hausmann, O.N., *Post-traumatic inflammation following spinal cord injury.* Spinal Cord, 2003. 41(7): p. 369-78.
30. Oyinbo, C.A., *Secondary injury mechanisms in traumatic spinal cord injury:a nugget of this multiply cascade.* Acta Neurobiologiae Experimentalis, 2011. 71: p. 19.
31. Hulsebosch, C.E., et al., *Mechanisms of chronic central neuropathic pain after spinal cord injury.* Brain Res Rev, 2009. 60(1): p. 202-13.
32. Chu, L.W., et al., *Atorvastatin prevents neuroinflammation in chronic constriction injury rats through nuclear NFkappaB downregulation in the dorsal root ganglion and spinal cord.* ACS Chem Neurosci, 2015. 6(6): p. 889-98.

33. Costa, B., et al., *AM404, an inhibitor of anandamide uptake, prevents pain behaviour and modulates cytokine and apoptotic pathways in a rat model of neuropathic pain.* Br J Pharmacol, 2006. 148(7): p. 1022-32.
34. Wagner, R., M. Janjigian, and R.R. Myers, *Anti-inflammatory interleukin-10 therapy in CCI neuropathy decreases thermal hyperalgesia, macrophage recruitment, and endoneurial TNF-α expression.* Pain, 1998. 74(1): p. 35-42.
35. Zhou, C., et al., *Montelukast attenuates neuropathic pain through inhibiting p38 mitogen-activated protein kinase and nuclear factor-kappa B in a rat model of chronic constriction injury.* Anesth Analg, 2014. 118(5): p. 1090-6.
36. Judith E. Kalinyak, R.I.D., Andrew R. Hoffman, and Andrew J. Perlman, *Tissue-specific Regulation of Glucocorticoid Receptor mRNA by Dexamethasone.* J Biol Chem, 1987. 262: p. 4.
37. Jamontt, J.M., et al., *The effects of Delta-tetrahydrocannabinol and cannabidiol alone and in combination on damage, inflammation and in vitro motility disturbances in rat colitis.* Br J Pharmacol, 2010. 160(3): p. 712-23.
38. Pertwee, R.G., *The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin.* Br J Pharmacol, 2008. 153(2): p. 199-215.
39. Bergamaschi, M.M., et al., *Safety and side effects of cannabidiol, a Cannabis sativa constituent.* Curr Drug Saf, 2011. 6(4): p. 237-49.
40. Ramer, R., et al., *COX-2 and PPAR-gamma confer cannabidiol-induced apoptosis of human lung cancer cells.* Mol Cancer Ther, 2013. 12(1): p. 69-82.
41. Yamaori, S., et al., *Characterization of major phytocannabinoids, cannabidiol and cannabinol, as isoform-selective and potent inhibitors of human CYP1 enzymes.* Biochem Pharmacol, 2010. 79(11): p. 1691-8.
42. Mantilla-Plata, B. and R.D. Harbison, *Distribution studies of (14C)delta-9-tetrahydrocannabinol in mice: effect of vehicle, route of administration, and duration of treatment.* Toxicol Appl Pharmacol, 1975. 34(2): p. 292-300.
43. Solowij, N., et al., *A protocol for the delivery of cannabidiol (CBD) and combined CBD and 9-tetrahydrocannabinol (THC) by vaporisation.* BMC Pharmacol Toxicol, 2014. 15: p. 58.
44. Ohlsson, A., et al., *Single-dose kinetics of deuterium-labelled cannabidiol in man after smoking and intravenous administration.* Biomed Environ Mass Spectrom, 1986. 13(2): p. 77-83.
45. Ellis, G.M., Jr., et al., *Excretion patterns of cannabinoid metabolites after last use in a group of chronic users.* Clin Pharmacol Ther, 1985. 38(5): p. 572-8.
46. Huestis, M.A. and E.J. Cone, *Differentiating new marijuana use from residual drug excretion in occasional marijuana users.* J Anal Toxicol, 1998. 22(6): p. 445-54.
47. Harvey, D.J. and R. Mechoulam, *Metabolites of cannabidiol identified in human urine.* Xenobiotica, 1990. 20(3): p. 303-20.
48. Ohlsson, A., et al., *Single-dose kinetics of deuterium-labelled cannabidiol in man after smoking and intravenous administration.* Biological Mass Spectrometry, 1986. 13(2): p. 77-83.
49. Agurell, S., et al., *Pharmacokinetics and metabolism of delta 1-tetrahydrocannabinol and other cannabinoids with emphasis on man.* Pharmacol Rev, 1986. 38(1): p. 21-43.

50. E Samara, M.B., R Mechoulam, *Pharmacokinetics of cannabidiol in dogs.* Drug Metabolism and Disposition, 1988. 16(3).
51. Karschner, E.L., et al., *Plasma cannabinoid pharmacokinetics following controlled oral delta9-tetrahydrocannabinol and oromucosal cannabis extract administration.* Clin Chem, 2011. 57(1): p. 66-75.
52. Lawrence, D.K. and R.G. Pertwee, *Brain levels of delta1-tetrahydrocannabinol and its metabolites in mice tolerant to the hypothermic effect of delta1-tetrahydrocannabinol.* Br J Pharmacol, 1973. 49(2): p. 373-5.
53. Deiana, S., et al., *Plasma and brain pharmacokinetic profile of cannabidiol (CBD), cannabidivarine (CBDV), Delta(9)-tetrahydrocannabivarin (THCV) and cannabigerol (CBG) in rats and mice following oral and intraperitoneal administration and CBD action on obsessive-compulsive behaviour.* Psychopharmacology (Berl), 2012. 219(3): p. 859-73.
54. Matsuda, L.A., et al., *Structure of a cannabinoid receptor and functional expression of the cloned cDNA.* Nature, 1990. 346(6284): p. 561-4.
55. Di Marzo, V., et al., *Enhancement of anandamide formation in the limbic forebrain and reduction of endocannabinoid contents in the striatum of delta9-tetrahydrocannabinol-tolerant rats.* J Neurochem, 2000. 74(4): p. 1627-35.
56. Munro, S., K.L. Thomas, and M. Abu-Shaar, *Molecular characterization of a peripheral receptor for cannabinoids.* Nature, 1993. 365(6441): p. 61-5.
57. Van Sickle, M.D., et al., *Identification and functional characterization of brainstem cannabinoid CB2 receptors.* Science, 2005. 310(5746): p. 329-32.
58. Balsevich, G., G.N. Petrie, and M.N. Hill, *Endocannabinoids: Effectors of glucocorticoid signaling.* Front Neuroendocrinol, 2017. 47: p. 86-108.
59. Bisogno, T., et al., *Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide.* Br J Pharmacol, 2001. 134(4): p. 845-52.
60. Glass, M. and C.C. Felder, *Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor.* J Neurosci, 1997. 17(14): p. 5327-33.
61. Gerachshenko, T., et al., *Presynaptic G-protein-coupled receptors dynamically modify vesicle fusion, synaptic cleft glutamate concentrations, and motor behavior.* J Neurosci, 2009. 29(33): p. 10221-33.
62. Lauckner, J.E., B. Hille, and K. Mackie, *The cannabinoid agonist WIN55,212-2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins.* Proc Natl Acad Sci U S A, 2005. 102(52): p. 19144-9.
63. Deshpande, L.S., R.E. Blair, and R.J. DeLorenzo, *Prolonged cannabinoid exposure alters GABA(A) receptor mediated synaptic function in cultured hippocampal neurons.* Exp Neurol, 2011. 229(2): p. 264-73.
64. Hajos, N., C. Ledent, and T.F. Freund, *Novel cannabinoid-sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus.* Neuroscience, 2001. 106(1): p. 1-4.
65. Merighi, S., et al., *Cannabinoid CB(2) receptors modulate ERK-1/2 kinase signalling and NO release in microglial cells stimulated with bacterial lipopolysaccharide.* Br J Pharmacol, 2012. 165(6): p. 1773-88.

66. Thomas, A., et al., *Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro.* Br J Pharmacol, 2007. 150(5): p. 613-23.
67. Hakimizadeh, E., et al., *Endocannabinoid System and TRPV1 Receptors in the Dorsal Hippocampus of the Rats Modulate Anxiety-like Behaviors.* Iran J Basic Med Sci, 2012. 15(3): p. 795-802.
68. Hofmann, M.E. and M.C. Andresen, *Vanilloids selectively sensitize thermal glutamate release from TRPV1 expressing solitary tract afferents.* Neuropharmacology, 2016. 101: p. 401-11.
69. Christoph, T., et al., *Silencing of vanilloid receptor TRPV1 by RNAi reduces neuropathic and visceral pain in vivo.* Biochem Biophys Res Commun, 2006. 350(1): p. 238-43.
70. Kanai, Y., et al., *Involvement of an increased spinal TRPV1 sensitization through its up-regulation in mechanical allodynia of CCI rats.* Neuropharmacology, 2005. 49(7): p. 977-84.
71. Zakir, H.M., et al., *Expression of TRPV1 channels after nerve injury provides an essential delivery tool for neuropathic pain attenuation.* PLoS One, 2012. 7(9): p. e44023.
72. Costa, B., et al., *Vanilloid TRPV1 receptor mediates the antihyperalgesic effect of the nonpsychoactive cannabinoid, cannabidiol, in a rat model of acute inflammation.* Br J Pharmacol, 2004. 143(2): p. 247-50.
73. Hegde, V.L., P.S. Nagarkatti, and M. Nagarkatti, *Role of myeloid-derived suppressor cells in amelioration of experimental autoimmune hepatitis following activation of TRPV1 receptors by cannabidiol.* PLoS One, 2011. 6(4): p. e18281.
74. Liu, D., W. Thangnipon, and D.J. McAdoo, *Excitatory amino acids rise to toxic levels upon impact injury to the rat spinal cord.* Brain Res, 1991. 547(2): p. 344-8.
75. Liu, D., et al., *Neurotoxicity of glutamate at the concentration released upon spinal cord injury.* Neuroscience, 1999. 93(4): p. 1383-9.
76. Xu, G.Y., et al., *Concentrations of glutamate released following spinal cord injury kill oligodendrocytes in the spinal cord.* Exp Neurol, 2004. 187(2): p. 329-36.
77. Hampson, A.J., et al., *Cannabidiol and (-)Delta9-tetrahydrocannabinol are neuroprotective antioxidants.* Proc Natl Acad Sci U S A, 1998. 95(14): p. 8268-73.
78. El-Remessy, A.B., et al., *Neuroprotective effect of (-)Delta9-tetrahydrocannabinol and cannabidiol in N-methyl-D-aspartate-induced retinal neurotoxicity: involvement of peroxynitrite.* Am J Pathol, 2003. 163(5): p. 1997-2008.
79. Wade, D.T., et al., *A preliminary controlled study to determine whether whole-plant cannabis extracts can improve intractable neurogenic symptoms.* Clin Rehabil, 2003. 17(1): p. 21-9.
80. Hallak, J.E.C., et al., *The interplay of cannabinoid and NMDA glutamate receptor systems in humans: Preliminary evidence of interactive effects of cannabidiol and ketamine in healthy human subjects.* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2011. 35(1): p. 198-202.
81. Gomes, F.V., et al., *Cannabidiol attenuates sensorimotor gating disruption and molecular changes induced by chronic antagonism of NMDA receptors in mice.* Int J Neuropsychopharmacol, 2015. 18(5).

82. Gobira, P.H., et al., *Cannabidiol, a Cannabis sativa constituent, inhibits cocaine-induced seizures in mice: Possible role of the mTOR pathway and reduction in glutamate release.* Neurotoxicology, 2015. 50: p. 116-21.
83. Vilela, L.R., et al., *Cannabidiol rescues acute hepatic toxicity and seizure induced by cocaine.* Mediators Inflamm, 2015. 2015: p. 523418.
84. Geffrey, A.L., et al., *Drug-drug interaction between clobazam and cannabidiol in children with refractory epilepsy.* Epilepsia, 2015. 56(8): p. 1246-51.
85. Tzadok, M., et al., *CBD-enriched medical cannabis for intractable pediatric epilepsy: The current Israeli experience.* Seizure, 2016. 35: p. 41-4.
86. Devinsky, O., et al., *Cannabidiol in patients with treatment-resistant epilepsy: an open-label interventional trial.* Lancet Neurol, 2016. 15(3): p. 270-8.
87. Frankfurt, M., E. Fuchs, and W. Wuttke, *Sex differences in gamma-aminobutyric acid and glutamate concentrations in discrete rat brain nuclei.* Neurosci Lett, 1984. 50(1-3): p. 245-50.
88. Ferri, S.L., et al., *Estradiol regulates markers of synaptic plasticity in the hypothalamic ventromedial nucleus and amygdala of female rats.* Horm Behav, 2014. 66(2): p. 409-20.
89. Honack, D. and W. Loscher, *Sex differences in NMDA receptor mediated responses in rats.* Brain Res, 1993. 620(1): p. 167-70.
90. Berdichevsky, E., et al., *Kainate, N-methylaspartate and other excitatory amino acids increase calcium influx into rat brain cortex cells in vitro.* Neurosci Lett, 1983. 36(1): p. 75-80.
91. Nicoll, R.A. and B.E. Alger, *Synaptic excitation may activate a calcium-dependent potassium conductance in hippocampal pyramidal cells.* Science, 1981. 212(4497): p. 957-9.
92. Shields, D.C., et al., *Calpain activity and expression increased in activated glial and inflammatory cells in penumbra of spinal cord injury lesion.* J Neurosci Res, 2000. 61(2): p. 146-50.
93. Khorchid, A. and M. Ikura, *How calpain is activated by calcium.* Nat Struct Biol, 2002. 9(4): p. 239-41.
94. Momeni, H.R. and M. Kanje, *Calpain inhibitors delay injury-induced apoptosis in adult mouse spinal cord motor neurons.* Neuroreport, 2006. 17(8): p. 761-5.
95. Zhang, Z., et al., *Therapeutic Efficacy of E-64-d, a Selective Calpain Inhibitor, in Experimental Acute Spinal Cord Injury.* Biomed Res Int, 2015. 2015: p. 134242.
96. Stout, A.K., et al., *Glutamate-induced neuron death requires mitochondrial calcium uptake.* Nat Neurosci, 1998. 1(5): p. 366-73.
97. Verweij, B.H., et al., *Mitochondrial dysfunction after experimental and human brain injury and its possible reversal with a selective N-type calcium channel antagonist (SNX-111).* Neurol Res, 1997. 19(3): p. 334-9.
98. White, R.J. and I.J. Reynolds, *Mitochondria and Na+/Ca2+ exchange buffer glutamate-induced calcium loads in cultured cortical neurons.* J Neurosci, 1995. 15(2): p. 1318-28.
99. Bartova, A. and M.K. Birmingham, *Effect of delta9-tetrahydrocannabinol on mitochondrial NADH-oxidase activity.* J Biol Chem, 1976. 251(16): p. 5002-6.

100. Sarafian, T.A., et al., *Delta 9-tetrahydrocannabinol disrupts mitochondrial function and cell energetics.* Am J Physiol Lung Cell Mol Physiol, 2003. 284(2): p. L298-306.
101. Harris, R.A. and J.A. Stokes, *Cannabinoids inhibit calcium uptake by brain synaptosomes.* J Neurosci, 1982. 2(4): p. 443-7.
102. Giudice, E.D., et al., *Cannabidiol, unlike synthetic cannabinoids, triggers activation of RBL-2H3 mast cells.* J Leukoc Biol, 2007. 81(6): p. 1512-22.
103. Ryan, D., et al., *Cannabidiol targets mitochondria to regulate intracellular Ca2+ levels.* J Neurosci, 2009. 29(7): p. 2053-63.
104. Drysdale, A.J., et al., *Cannabidiol-induced intracellular Ca2+ elevations in hippocampal cells.* Neuropharmacology, 2006. 50(5): p. 621-31.
105. Hains, B.C. and S.G. Waxman, *Activated microglia contribute to the maintenance of chronic pain after spinal cord injury.* J Neurosci, 2006. 26(16): p. 4308-17.
106. Gensel, J.C. and B. Zhang, *Macrophage activation and its role in repair and pathology after spinal cord injury.* Brain Res, 2015. 1619: p. 1-11.
107. Pruss, H., et al., *Non-resolving aspects of acute inflammation after spinal cord injury (SCI): indices and resolution plateau.* Brain Pathol, 2011. 21(6): p. 652-60.
108. Popovich, P.G., P. Wei, and B.T. Stokes, *Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats.* J Comp Neurol, 1997. 377(3): p. 443-64.
109. Vuolo, F., et al., *Evaluation of Serum Cytokines Levels and the Role of Cannabidiol Treatment in Animal Model of Asthma.* Mediators Inflamm, 2015. 2015: p. 538670.
110. Faden, A.I., A. Gannon, and A.I. Basbaum, *Use of serotonin immunocytochemistry as a marker of injury severity after experimental spinal trauma in rats.* Brain Res, 1988. 450(1-2): p. 94-100.
111. Hashimoto, T. and N. Fukuda, *Contribution of serotonin neurons to the functional recovery after spinal cord injury in rats.* Brain Res, 1991. 539(2): p. 263-70.
112. Landry, E.S., et al., *Contribution of spinal 5-HT1A and 5-HT7 receptors to locomotor-like movement induced by 8-OH-DPAT in spinal cord-transected mice.* Eur J Neurosci, 2006. 24(2): p. 535-46.
113. Lalley, P.M., *Serotoninergic and non-serotoninergic responses of phrenic motoneurones to raphe stimulation in the cat.* J Physiol, 1986. 380: p. 373-85.
114. Yaksh, T.L. and P.R. Wilson, *Spinal serotonin terminal system mediates antinociception.* J Pharmacol Exp Ther, 1979. 208(3): p. 446-53.
115. Crisp, T., et al., *Serotonin contributes to the spinal antinociceptive effects of morphine.* Pharmacol Biochem Behav, 1991. 39(3): p. 591-5.
116. Russo, E.B., et al., *Agonistic properties of cannabidiol at 5-HT1a receptors.* Neurochem Res, 2005. 30(8): p. 1037-43.
117. Ward, S.J., et al., *Cannabidiol inhibits paclitaxel-induced neuropathic pain through 5-HT(1A) receptors without diminishing nervous system function or chemotherapy efficacy.* Br J Pharmacol, 2014. 171(3): p. 636-45.
118. Hains, B.C., et al., *Changes in serotonin, serotonin transporter expression and serotonin denervation supersensitivity: involvement in chronic central pain after spinal hemisection in the rat.* Exp Neurol, 2002. 175(2): p. 347-62.

119. Sharma, H.S., et al., *Prostaglandins modulate alterations of microvascular permeability, blood flow, edema and serotonin levels following spinal cord injury: an experimental study in the rat.* Neuroscience, 1993. 57(2): p. 443-9.
120. Liu, D.X., et al., *Norepinephrine and serotonin release upon impact injury to rat spinal cord.* J Neurotrauma, 1990. 7(4): p. 219-27.
121. De Backer, O., et al., *Peroxisome proliferator-activated receptor gamma activation alleviates postoperative ileus in mice by inhibition of Egr-1 expression and its downstream target genes.* J Pharmacol Exp Ther, 2009. 331(2): p. 496-503.
122. Fahmi, H., J.P. Pelletier, and J. Martel-Pelletier, *PPARgamma ligands as modulators of inflammatory and catabolic responses in arthritis. An overview.* J Rheumatol, 2002. 29(1): p. 3-14.
123. Shi, Y., M. Hon, and R.M. Evans, *The peroxisome proliferator-activated receptor delta, an integrator of transcriptional repression and nuclear receptor signaling.* Proc Natl Acad Sci U S A, 2002. 99(5): p. 2613-8.
124. Luo, Y., et al., *PPAR-alpha and PPAR-beta expression changes in the hippocampus of rats undergoing global cerebral ischemia/reperfusion due to PPAR-gamma status.* Behav Brain Funct, 2014. 10(1): p. 21.
125. Quintanilla, R.A., E. Utreras, and F.A. Cabezas-Opazo, *Role of PPAR gamma in the Differentiation and Function of Neurons.* PPAR Res, 2014. 2014: p. 768594.
126. Kadowaki, K., et al., *Sex differences in PPARgamma expressions in rat adipose tissues.* Biol Pharm Bull, 2007. 30(4): p. 818-20.
127. Liu, Y., et al., *PPARgamma mRNA in the adult mouse hypothalamus: distribution and regulation in response to dietary challenges.* Front Neuroanat, 2015. 9: p. 120.
128. Mattern, H.M., et al., *Gender and genetic differences in bladder smooth muscle PPAR mRNA in a porcine model of the metabolic syndrome.* Mol Cell Biochem, 2007. 302(1-2): p. 43-9.
129. Morgenweck, J., et al., *Activation of peroxisome proliferator-activated receptor gamma in brain inhibits inflammatory pain, dorsal horn expression of Fos, and local edema.* Neuropharmacology, 2010. 58(2): p. 337-45.
130. Woster, A.P. and C.K. Combs, *Differential ability of a thiazolidinedione PPARgamma agonist to attenuate cytokine secretion in primary microglia and macrophage-like cells.* J Neurochem, 2007. 103(1): p. 67-76.
131. Xu, L., et al., *25-Hydroxycholesterol-3-sulfate attenuates inflammatory response via PPARgamma signaling in human THP-1 macrophages.* Am J Physiol Endocrinol Metab, 2012. 302(7): p. E788-99.
132. Zhang, M.A., et al., *Peroxisome proliferator-activated receptor (PPAR)alpha and -gamma regulate IFNgamma and IL-17A production by human T cells in a sex-specific way.* Proc Natl Acad Sci U S A, 2012. 109(24): p. 9505-10.
133. Fang, I.M., C.H. Yang, and C.M. Yang, *Docosahexaenoic acid reduces linoleic acid induced monocyte chemoattractant protein-1 expression via PPARgamma and nuclear factor-kappaB pathway in retinal pigment epithelial cells.* Mol Nutr Food Res, 2014. 58(10): p. 2053-65.
134. Kapadia, R., J.H. Yi, and R. Vemuganti, *Mechanisms of anti-inflammatory and neuroprotective actions of PPAR-gamma agonists.* Front Biosci, 2008. 13: p. 1813-26.

135. Xu, J., et al., *Methylprednisolone inhibition of TNF-alpha expression and NF-kB activation after spinal cord injury in rats.* Brain Res Mol Brain Res, 1998. 59(2): p. 135-42.
136. Duan, S.Z., M.G. Usher, and R.M. Mortensen, *Peroxisome proliferator-activated receptor-gamma-mediated effects in the vasculature.* Circ Res, 2008. 102(3): p. 283-94.
137. Antonelli, A., et al., *Extra-ocular muscle cells from patients with Graves' ophthalmopathy secrete alpha (CXCL10) and beta (CCL2) chemokines under the influence of cytokines that are modulated by PPARgamma.* Autoimmun Rev, 2014. 13(11): p. 1160-6.
138. Vallabhapurapu, S. and M. Karin, *Regulation and function of NF-kappaB transcription factors in the immune system.* Annu Rev Immunol, 2009. 27: p. 693-733.
139. KEANE, R.W., KRAYDIEH, S., LOTOCKI, G., BETHEA, J.R., KRAJEWSKI, S., REED, J.C., AND DIETRICH, W.D., *Apoptotic and anti-apoptotic mechanisms following spinal cord injury.* J Neuropathol Exp Neuro, 2001. 60(5): p. 8.
140. Rafati, D.S., et al., *Nuclear factor-kappaB decoy amelioration of spinal cord injury-induced inflammation and behavior outcomes.* J Neurosci Res, 2008. 86(3): p. 566-80.
141. Scuderi, C., L. Steardo, and G. Esposito, *Cannabidiol promotes amyloid precursor protein ubiquitination and reduction of beta amyloid expression in SHSY5YAPP+ cells through PPARgamma involvement.* Phytother Res, 2014. 28(7): p. 1007-13.
142. O'Sullivan, S.E., et al., *Time-dependent vascular actions of cannabidiol in the rat aorta.* Eur J Pharmacol, 2009. 612(1-3): p. 61-8.
143. Maynard, F.M., Jr., et al., *International Standards for Neurological and Functional Classification of Spinal Cord Injury. American Spinal Injury Association.* Spinal Cord, 1997. 35(5): p. 266-74.
144. Fehlings, M.G. and R.G. Perrin, *The timing of surgical intervention in the treatment of spinal cord injury: a systematic review of recent clinical evidence.* Spine (Phila Pa 1976), 2006. 31(11 Suppl): p. S28-35; discussion S36.
145. Sekhon, L.H. and M.G. Fehlings, *Epidemiology, demographics, and pathophysiology of acute spinal cord injury.* Spine (Phila Pa 1976), 2001. 26(24 Suppl): p. S2-12.
146. Mirza, S.K., et al., *Early versus delayed surgery for acute cervical spinal cord injury.* Clin Orthop Relat Res, 1999(359): p. 104-14.
147. Chaovipoch, P., et al., *17beta-estradiol is protective in spinal cord injury in post- and pre-menopausal rats.* J Neurotrauma, 2006. 23(6): p. 830-52.
148. Kachadroka, S., et al., *Effect of endogenous androgens on 17beta-estradiol-mediated protection after spinal cord injury in male rats.* J Neurotrauma, 2010. 27(3): p. 611-26.
149. Olsen, M.L., et al., *Spinal cord injury causes a wide-spread, persistent loss of Kir4.1 and glutamate transporter 1: benefit of 17 beta-oestradiol treatment.* Brain, 2010. 133(Pt 4): p. 1013-25.
150. Siriphorn, A., S. Chompoopong, and C.L. Floyd, *17beta-estradiol protects Schwann cells against H2O2-induced cytotoxicity and increases transplanted Schwann cell survival in a cervical hemicontusion spinal cord injury model.* J Neurochem, 2010. 115(4): p. 864-72.

151. Sribnick, E.A., et al., *Estrogen attenuated markers of inflammation and decreased lesion volume in acute spinal cord injury in rats.* J Neurosci Res, 2005. 82(2): p. 283-93.
152. Yune, T.Y., et al., *Systemic administration of 17beta-estradiol reduces apoptotic cell death and improves functional recovery following traumatic spinal cord injury in rats.* J Neurotrauma, 2004. 21(3): p. 293-306.
153. Carlsson, M. and A. Carlsson, *A regional study of sex differences in rat brain serotonin.* Prog Neuropsychopharmacol Biol Psychiatry, 1988. 12(1): p. 53-61.
154. Jovanovic, H., et al., *Sex differences in the serotonin 1A receptor and serotonin transporter binding in the human brain measured by PET.* Neuroimage, 2008. 39(3): p. 1408-19.
155. Ueki, S., et al., *Regulation of peroxisome proliferator-activated receptor-gamma expression in human eosinophils by estradiol.* Int Arch Allergy Immunol, 2009. 149 Suppl 1: p. 51-6.
156. Mouton, P.R., et al., *Age and gender effects on microglia and astrocyte numbers in brains of mice.* Brain Res, 2002. 956(1): p. 30-5.
157. Sorge, R.E., et al., *Different immune cells mediate mechanical pain hypersensitivity in male and female mice.* Nat Neurosci, 2015. 18(8): p. 1081-3.
158. Ashcroft, G.S., et al., *Estrogen modulates cutaneous wound healing by downregulating macrophage migration inhibitory factor.* J Clin Invest, 2003. 111(9): p. 1309-18.
159. Crown, E.D., et al., *Calcium/calmodulin dependent kinase II contributes to persistent central neuropathic pain following spinal cord injury.* Pain, 2012. 153(3): p. 710-21.
160. Ellis, A., et al., *Systemic administration of propentofylline, ibudilast, and (+)-naltrexone each reverses mechanical allodynia in a novel rat model of central neuropathic pain.* J Pain, 2014. 15(4): p. 407-21.
161. Angelika EM Mautes, M.R.W., Frances Donovan, Linda J Noble, *Vascular Events After Spinal Cord Injury: Contribution to Secondary Pathogenesis.* Phys Ther, 2000. 80: p. 17.
162. Charles H. tator, M.G.F., *Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms.* J Neurosurg, 1991. 75: p. 12.
163. Emil Samara, M.B., and Raphael Mechoulam, *Pharmacokinetics of cannabidiol in dogs.* Drug Metabolism and Disposition, 1988. 16(3): p. 4.
164. Berman, J. *A Study of Cannabis Based Medicine Extracts and Placebo in Patients With Pain Due to Spinal Cord Injury*. 2012 August 2012; Available from: https://clinicaltrials.gov/ct2/show/study/NCT01606202?term=spinal+cord+injury+AND+Neuropathic+Pain&no_unk=Y&cond=%22Spinal+Cord+Injuries%22&rank=24§=X43kjihgfedcba8970156.

CANNABIDIOL ADMINISTRATION AFTER SPINAL CORD INJURY REDUCES ALLODYNIA IN BOTH MALE AND FEMALE RATS, WITH MOST ROBUST EFFECTS IN FEMALES

by

AMANDA MOHAIMANY-APONTE, BETTY M. PAT, SEAN D. MCALLISTER, AND CANDACE L. FLOYD

In preparation for *Journal of Neurotrauma*

Format adapted for dissertation

Cannabidiol administration after spinal cord injury reduces allodynia in both male and female rats, with most robust effects in females

Amanda Mohaimany-Aponte[1], Betty M. Pat, Ph.D.[1], Sean D. McAllister, Ph.D.[2], and Candace L. Floyd, Ph.D.[1]

[1]University of Alabama at Birmingham, Birmingham, AL and [2]California Pacific Medical Center Research Institute, San Francisco, CA

Abstract

Chronic, neuropathic pain development post-spinal cord injury (SCI-NP) affects a vast majority of patients and lowers quality of life as there are few effective therapies for this condition. Clinically, cannabidiol (CBD) has had success in treating NP disorders and has been shown to have pleiotropic effects on many aspects that contribute to SCI-NP. The effects of CBD treatment on SCI-NP remain unknown, therefore the purpose of this study was to examine the therapeutic potential of acute administration of CBD on chronic outcomes post-SCI in male and female rats. Prior to surgery, baseline measures of motor and sensory function were taken. Animals were divided into surgical control (LAM) or injury (fifth vertebral level cervical unilateral injury). Thirty minutes post-surgery, animals received an intraperitoneal injection of either vehicle (Veh) or CBD, continuing once per day for 7 consecutive days. CBD treatment prevented diminished gains in weight post-SCI in males and exhibited short-lived gains in motor function in females. Beyond this, CBD administration reduced autophagic behavior in both sexes and significantly reduced cold-induced sensitivity sub-acutely in males and chronically in

females. Utilizing the Grimace Assessment, it was observed that facial grimace scores increased after acetone application in SCI animals. Histologically, SCI significantly reduced neuronal numbers and white matter area at the site of injury, with CBD administration having no effect on neuronal numbers. In regards to white matter, SCI Veh females exhibited greater white matter sparing compared to males, additionally, females who received CBD treatment had significantly less white matter compared to non-treated SCI females. These results indicate that CBD has some therapeutic efficacy in both sexes, but confers greater protection in females.

Introduction

Over 300,000 individuals are living with chronic spinal cord injury (SCI) in the United States with an annual incidence of over 17,000 cases with the most common type being incomplete cervical injury [1]. Within the SCI patient population, between 60-80% go on to develop neuropathic pain (SCI-NP), a persistent issue that develops chronically post-SCI [2-4]. SCI-NP development has been shown to significantly diminish quality of life via increased incidence of depression, anxiety, and decreased work capacity [2, 5, 6]. Exacerbating the issue of diminished quality of life is few treatment options and management of pain episodes [2, 4, 5, 7, 8].

Within the context of pain disorders, a majority of pain patients are female, with female SCI patients reporting greater incidence and quality of pain compared to male SCI patients [3, 7]. Unique to SCI, a majority of patients are male, roughly 80% [1]. Considering these factors, it is highly important to study both sexes when evaluating SCI-

NP, as sex has been shown to affect therapeutic efficacy and mechanisms contributing to pain development [9-12].

SCI-NP manifests itself in two phenotypes, allodynia and hyperalgesia, which can present themselves either separately or in conjunction in SCI patients through a variety of stimuli including touch, cold, or heat [6, 13-16]. Abnormal nerve signaling from the site of injury is the root cause of NP [17, 18]. In the context of SCI, primary injury is the initial source of injury however secondary injury (2^{O} injury) has shown to greatly contribute to SCI-NP development and maintenance [19-23]. Past studies have shown that 2^{O} injury begins acutely after SCI and that acute targeting can affect chronic outcomes post injury [11, 24-26]. Though therapeutics have had success in animals models of SCI-NP, few therapeutics have had clinical efficacy in treating SCI-NP [6, 15, 27].

Clinically, cannabidiol (CBD) has been used successfully to treat NP stemming from a variety of disorders including multiple sclerosis and fibromyalgia in both males and females [28-31]. CBD, a component of *Cannabis sativa* (*C. sativa*), elicits little to no psychotropic effects and few to no adverse effects while being able to penetrate into the central nervous system (CNS) [32-35]. Mechanistically, CBD exhibits anti-inflammatory, anti-oxidant, and neuroprotective effects which have been shown to begin acutely after SCI and contribute to SCI-NP development at chronic time points post injury [19, 20, 22-24, 36-52]. However, the potential therapeutic efficacy of CBD within the SCI-NP patient population remains unelucidated. The purpose of this study was to determine the effect of acute CBD treatment on chronic outcomes post-SCI in both male and female rats.

Material and Methods

Animals

Male and female Sprague Dawley rats (Charles River) aged 2 – 3 months were used. Animals were group housed 2-3 to a cage in a temperature, humidity, and light (12 hr: 12 hr light dark cycle) controlled facility and provided food and water *ad libitum*. Prior to behavioral testing, all animals were handled once daily for 5 consecutive days for 5 minutes, during which time they were stroked from head to tail. After handling, animals were acclimated to behavioral equipment by placing the animal in the apparatus sans testing for 10 minutes for a duration of once per day for 5 consecutive days. Post habituation, baseline (BL) measures were taken and animals were randomly and blindly divided into surgical control (laminectomy, LAM) or SCI. SCI groups given either vehicle (VEH) or drug (CBD). Within males and females, there were three groups: LAM, SCI VEH, and SCI CBD with n's ranging from 10-13 per group.

On weeks 1, 3, and 5 post-surgery, animals were assessed on motor function and on weeks 2, 4, and 6, animals were assessed on pain. Additionally, all animals were observed twice daily with body mass, animal welfare, and autophagic phenotype noted. Animals exhibiting hindpaw nail chewing and/or digit/paw swelling for three consecutive days or longer with no abatement in phenotype were deemed as exhibiting autophagic behavior. At termination of the study, all animals were euthanized and tissue was collected for histological analysis. All protocols done were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and in concurrence with the National Institutes of Health guidelines on animal research.

Surgery

All animals were placed under anesthesia using a 4% isoflurane and 2% oxygen gas mixture for 4 minutes, and then placed under a 2% isoflurane and 2% oxygen gas mixture for maintenance of anesthesia during the surgical procedure. Heating pads in conjunction with rectal probe were used to maintain body temperature $37^{0}C \pm 1^{0}C$ throughout the duration of the procedure. The dorsal region of the back was shaved from the top of the shoulder blades to mid-back. Exposed skin was then cleaned using chlorhexidine and benzadine and eye lubricant was placed up the eyes. The cervical region of the back and muscle layers were incised to expose the dorsal region of the cervical vertebrae. Using morphological features, the 5th cervical vertebral level (C5) was identified, and verified by another surgeon. Post verification, the C5 was dorsally laminectomized and verified. The animal was then placed on a stereotaxic platform with clamping occurring at C1 and thoracic vertebrae level 2 (T2). The animal was then placed in the impactor device (Infinite Horizon SCI Device, Precision Systems and Instrumentation; Lexington, Kentucky) and the impactor tip was positioned above either the left or right side of the exposed C5 spinal cord, determined by dominant paw. Placement was verified and body temperature was recorded. Animals randomly designated as surgical laminectomy controls (LAM), were halted at this point and received no impact. Animals in the SCI group received a unilateral 300 kdyne impact at the C5 region of the spinal cord. Animals were then removed from the stereotaxic platform, sutured, and placed in a recovery cage and observed for ambulation before being returned to the group housed cage. Post-surgery, animals received a subcutaneous injection of saline, rimadyl, and antibiotic (Bayer Healthcare LLC, Baytril ®, enrofloxacin) for three days.

Drug Treatment

Animals were randomly assigned into their respective treatment groups, with the individual performing behavioral assessments and those delivering treatment blinded to the animal treatment status. At 30 minutes post-surgery then once per day for seven consecutive days, an intraperitoneal (i.p.) injection of either VEH or drug (CBD) was given at a dose of 100 mg/kg/mL. CBD was dissolved in a mixture of saline, tween-80, and ethanol at a 1:1:4 ratio, VEH contained no CBD.

CBD Plasma Concentration

Animals were weighed and given an i.p. injection of CBD, formulated as described above, at a 0, 1, 10, and 100 mg/kg/mL dose (n=2 per dose). One hour post-injection, animals were euthanized and had blood drawn. The blood was centrifuged for 15 minutes at 2000 rpm and the serum was extracted. Serum then underwent protein precipitation where 40 uL sample plus twice the volume of ice cold Internal Standard Solution (acetonitrile containing 200 ng/mL cannibidiol-D3). Samples were then centrifuged at 6100g for 30 minutes and an aliquot of each supernatant was transferred to an autosampler plate with glass inserts and diluted with one volume of 0.2% formic acid in water. Samples were then injected into a Higgins analytical column (injection volume of 20 uL) at room temperature with mobile phase A being 0.2% formic acid in water and mobile phase B being 0.2% formic acid in acetonitrile. The high-performance liquid chromatographer (HPLC; Shimadzu VP series 10 system) utilized an electrospray ionization method with transitions (m/z) of CBD (315.3/193.3) and CBD-*D3* (318.3/196.3) specified.

Behavior

Measures of motor and sensory function occurred prior to surgery and post-surgery once weekly for 6 weeks. Motor function tests were taken on weeks 1, 3, and 5 post-surgery, and sensory function measures were taken on weeks 2, 4, and 6. After placement in the sensory function testing device, all animals were given 5 minutes to acclimate to the apparatus. Males and females were assessed on separate days.

Motor Function: Paw Placement

Animals were put in a Plexiglas cylinder (40 cm height, 30 cm diameter) for 5 minutes, during which time the number of forepaw placements with body weight support were counted as either right, left, or both. Determination of dominant paw was performed using baseline assessments, with a minimum number of 25 total paw placements set. Percent of right and left placements were determined using the following equation: % Right = (Right placements)/ (Summed total of placements) * 100.

Mechanical Allodynia: Von Frey

Animals were placed on a platform with a wire mesh bottom and enclosed using a Plexiglas box (21.5 cm length x 12.5 cm width x 12.5 cm height) and given five minutes to acclimate. Von Frey filaments (Touch Test ®, North Coast Medical and Rehabilitation Products), beginning at 2 grams (g) with a terminal filament of 15 g, were applied to the plantar region of the hindpaw for five seconds, five separate times using the up/down method. The Dixon score was then calculated using the final filament using the following equation: $Xf + kd$; Xf final filament force, k: constant; average difference of force between filaments, d: tabular value based on pattern of paw responses.

Cold Allodynia: Acetone Test

Animals were set on an elevated platform with a wire mesh bottom and enclosed in a Plexiglas box (21.5 cm length x 12.5 cm width x 12.5 cm height), then allowed five minutes to acclimate. Using a blunt ended syringe, a bubble of acetone was touched to the plantar region of the hindpaw without mechanical force. The animal was then observed for 20 seconds (s) for a paw withdrawal to the stimuli. Each paw was assessed three separate times with an interval of 5 minutes between assessments. The percent of paw withdrawal was then calculated for each hindpaw.

Supraspinal Aspect of Pain: Grimace Assessment

During the Acetone Test, animals were video recorded (Casio Exilim, high definition, 60 frames per second) for 30 s prior to and 30 s after acetone application, with a mirror placed behind the animal to ensure recording during animal movement or turning. Still frames were collected, one still frame per second, beginning at 20 s prior to acetone application and ending at 20 seconds after application for a total sum of 40 still frames per acetone trial. Still frames consisted of a clear picture of the rodent face, containing facial action units (AU) by which the picture would be assessed on. Four AU were utilized, orbital tightening, nose/cheek flattening, ear changes, and whisker changes.

After still collection at BL (n=10) and Week 6 (n= 10-11), stills were randomly sorted into a slide show and recoded. Individuals performing still scoring assessed each AU and scored them as either 0, 1, or 2. A score of 0 indicated no observable action, 1 indicated slight/moderate observable action, and a 2 indicated robust/pronounced observable action. The AU average was then calculated using the scores of two individual scorers.

Total facial grimace score was determined by averaging the four AU's. Total facial score was determined before and after acetone application.

To ensure proper blinding during filming, still collection, still randomization, still scoring, and analysis, animal sex, surgery assignment, treatment status, and stage of study was not revealed to the individuals collecting still frames nor the individuals randomizing the stills. In addition, the individuals performing these tasks did not participate in animal filming or behavioral assessment. Beyond this, the individual filming and the individual performing the assessment were also blinded to the surgery assignment and treatment status of the animal. The individuals randomizing the stills recoded the file name to ensure those scoring the stills remained blinded to the identity of the animal. The two individuals scoring the stills had an inter-rater reliability (IRR) an overall score of 96%, with a range of 91% - 99% on individual AU IRR with 123 still frames scored.

Tissue Analysis

At Week 6, animals were euthanized via exsanguination with intracardial perfusion using cold, 0.1 molar (M) phosphate buffered saline (PBS; 7.4 pH) for 5 minutes and subsequent perfusion with fixative (American MasterTech Scientific, Inc. ExCell PLUS ™) for 10 minutes. Post-fixative perfusion, the cervical region of the spinal cord was removed and place in fixative for seven days at 4^0 C. After fixation, the cervical tissue was subjected to water removal via sucrose gradient exposure, 10% sucrose PB solution for one hour at 4°C, then 30% sucrose PB solution for 48 hours at 4°C. Cervical tissue was then cut into three sections, caudal to epicenter, epicenter, and rostral to epicenter,

each three millimeters (mm) in length. Tissue was then placed in embedding medium and stored at -80°C until sliced.

Tissue was sliced transversely into 30 µm serial sections containing the areas rostral to, at, and caudal to epicenter using a cryostat machine (Leica Biosystems CM 1860) and placed on a glass slide coated with 1% gelatin and chrom alum. Serial sections were mounted in sets of ten; therefore an interval of 300 µm existed between adjacently mounted slices meaning each slide represented 2100 µm of tissue. Slides were stored at -20°C until ready for staining. Post-staining, tissue was quantified using an Olympus IX73 microscope with Visiopharm® imaging software.

Neuronal Numbers: Cresyl Violet

Post-mounting, tissue was dehydrated, defatted, and rehydrated. Dehydration occurred by tissue placement in series of ethanol solutions of increasing gradient, 50%, 70%, 95%, and 100% for 2 minutes at each concentration. After dehydration, tissue was defatted via xylene exposure at 5 minutes, 10 minutes, and 1 minute. Tissue was then rehydrated via re-exposure to ethanol solutions at decreasing gradient, 100%, 95%, and 70%, for 1 minute in each concentration, then two water baths for 30 seconds each. Post-rehydration, tissue was stained with cresyl violet acetate for 4 minutes, rinsed in a water bath, then placed in 95% ethanol with acetic acid for 2.5 minutes to differentiate staining nissl from non-nissl substances. Stained tissue underwent another dehydration process at 95% and 100% ethanol solutions for 30 s each and xylene baths at 5 minutes each. Following xylene bath exposure, slides were cover-slipped with mounting media and allowed to dry. Stained tissue was stored at room temperature until quantification.

Spinal cord sections were divided into two sections, ipsilateral to injury and contralateral to injury. The ventral area of the gray matter on these areas was traced. Utilizing the Visiopharm® imaging software, the regions of interest were quantified, with 40% of the region randomly counted for motor neurons.

White Matter Sparing: Eriochrome Cyanine R

After slide mounting, tissue was subjected to dehydration, defatting, and rehydration. Tissue was dehydrated via submersion in ethanol solutions of increasing concentration, 50%, 70%, 95%, and 100% for 3 minutes each. Following dehydration, tissue was defatted via xylene baths for 5 minutes, 10 minutes, and 1 minute. Post-defatting, tissue was rehydrated via baths in ethanol solutions of decreasing concentration, 100%, 95%, 70%, and 50% for 1 minute each followed by two water baths for 30 s each. After rehydration, tissue was placed in a 0.2% eriochrome cyanine R solution for 10 minutes, then rinsed in a water bath and placed in a 0.5% ammonia hydroxide solution for 1 minute in order to differentiate oligodendrocytes complexed with the eriochrome cyanine R stain, then rinsed in a water bath. The stained tissue was then dehydrated using ethanol solutions of increasing concentration, 95% and 100% for 30 s followed by two xylene baths for 5 minutes each. The slides were then cover-slipped using mounting media and allowed to dry and stored at room temperature.

The stained slices were divided medially into the area ipsilateral to injury and contralateral to injury. Using the Visiopharm® imaging software, the unilateral area was traced and the area calculated. Within the same unilateral area, the gray matter was traced and the area calculated. The gray matter area was then subtracted from the total area to

reveal the area of the white matter. The area of the white matter was then divided by the total area to calculate the percent area of the white matter. This was performed for the area ipsilateral and contralateral to injury.

Data Analysis

Data was analyzed using GraphPad Prism software 7.03. Data shown represents the mean ± the standard error of the mean, with significance set at $p \leq 0.05$. All data was analyzed using 2-way ANOVA. The symbols * represents significance between SCI and LAM groups, ^ represents significance between CBD and VEH groups, # represents significance between male and female groups, and & representing significance between ipsilateral and contralateral regions of the spinal cord.

Results

CBD Plasma Concentration

One hour post i.p. injection, blood was drawn and plasma extracted then processed through an HPLC system to calculate the concentration (ng/mL) of CBD present. A logarithmic dose injection scheme was used. It was found that concentrations of plasma CBD reflected values observed in previous literature (figure 1; Fig. 1).

Body Mass

Animals had a BL weight obtained prior to surgery, and were weighed once daily after surgery until the end of the study. At weeks 3, 4, and 6 post-surgery, it was observed that male SCI Veh treated animals had significantly lower gains in body mass (g) compared to LAM animals (Fig 2A). Animals treated with CBD were not significantly different from non-treated SCI animals or LAM animals. At Weeks 2 and 5 post injury, male SCI Veh animals trended on diminished gains in mass ($p = 0.0644$ and 0.0537 respectively) There was no observed effect of injury or drug treatment in female animals (Fig. 2B).

Motor Function: Paw Placement

Prior to surgery, the dominant paw (side ipsilateral to injury) was determined utilizing paw placement with a minimum of 25 total touches. It was observed that both sexes utilized their dominant paw approximately 60% of the time at BL, with LAM animals remaining statistically similar to their respective BL value throughout the duration of the study (Fig. 3 A and B). After SCI, there was a marked decrease in dominant paw use in both males and females which persisted until the end of the study. At week 3, female SCI animals treated with CBD had significantly greater dominant paw use compared to untreated SCI animals and at week 5 this effect was no longer present (Fig. 3B). There was no significant effect of CBD treatment observed in males, nor a sex effect.

Mechanical Sensory Function: Von Frey

Before surgery, BL measures of mechanical sensory function were assessed using von Frey. The mechanical threshold (g) was calculated using the Dixon Score. All animals were statistically similar at BL, with LAM male and female animals remaining similar to BL throughout the duration of the study. Both SCI groups, regardless of sex and drug treatment, remained statistically similar to their respective control groups (Fig 4. A and B).

Cold Sensory Function: Acetone Test

At BL, all animals were statistically similar to one another, with an incidence of paw withdrawal (hind paw contralateral to injury) at approximately 6%, with all LAM animals remaining similar to BL and each other at all time points after surgery. Both male and female SCI Veh treated animals, beginning at Week 2 and persisting until the end of the study, had significantly greater incidence of paw withdrawal compared to surgical controls (Fig. 5 A and B). It was also observed that male SCI CBD treated animals had significantly greater incidence of paw withdrawal compared to surgical controls at Weeks 2 and 6, during these time points male SCI CBD animals were statistically similar to SCI Veh treated animals (Fig. 5 A). At Week 4, CBD treated males had significantly less incidence of paw withdrawal compared to non-treated SCI animals and were statistically similar to LAM animals, however at Week 6, this effect was no longer present (Fig. 5 A).

Similar to male SCI Veh treated animals, beginning at Week 2 and continuing through the end of the study, had greater incidence of paw withdrawal compared to LAM animals. Female SCI CBD treated animals were not statistically different from LAM animals at

Weeks 2 and 4, additionally at Week 4, SCI CBD animals had significantly less incidence of paw withdrawal compared to SCI Veh animals (Fig. 5 B). At Week 6, SCI CBD treated animals exhibited greater incidence of paw withdrawal compared to LAM animals (Fig. 5 B). Upon comparison, there was no significant effect of sex observed throughout the entire study.

Grimace Assessment

During the Acetone test animals were recorded in order to assess supraspinal responses to cold stimuli. At Week 6 post surgery, uninjured animals had a facial score similar to BL indicating no change in supraspinal response to cold stimuli (Fig. 6). Additionally, it was observed that facial scores before application of acetone was similar between all groups and to BL. After acetone application, both female SCI groups exhibited an increase in grimace facial score compared to scores before application and their respective LAM group in regards to grimace facial score after acetone exposure. Regarding males, an increase in grimace facial score was seen in male SCI CBD animals compared to their facial scores before acetone application. Male SCI Veh animals trended towards an increased facial grimace score compared to scores before acetone application ($p = 0.1106$). No differences were observed between SCI groups nor sex.

Autophagic Behavior

All animals were observed twice daily during which time any observations of nail chewing, nail and bleeding, skin irritation, digit swelling, and digit penetrance. Observations persisting for 3 consecutive days with no resolution or improvement

resulted in animals being deemed as exhibiting autophagic behavior. All SCI groups exhibited autophagic behavior, LAM animals exhibited no autophagic behavior (data not shown). Approximately 55% of male SCI Veh treated animals (6 out of 11 animals) and 46% of female SCI Veh treated animals (6 out of 13 animals) exhibited autophagic behavior (Fig. 7 A and B). Male and female SCI CBD treated groups exhibited lower incidences of autophagia (30%, 3 out of 10 male animals; 18%, 2 out of 11 female animals; Fig. 7 C and D).

Cresyl Violet: Neuronal Counts

Utilizing a cresyl violet stain to stain Nissl substance, the neurons in the ventral horn ipsilateral and contralateral to injury were quantified. Surgical controls exhibited no SCI pathology, while all SCI animals exhibited pathology unilateral to injury regardless of sex or treatment (Fig. 8 A). Neuron counts quantified in surgical controls, with no sex differences or region differences observed. The ventral horn contralateral to injury on all SCI groups, regardless of sex, were statistically similar to the surgical control groups (Fig. 8 C). The ventral horn ipsilateral to injury had significantly less neuronal numbers compared to the region contralateral to injury (Fig. 8 B). There was no observed effect of CBD treatment on neuronal counts.

Eriochrome Cyanine R: White Matter Sparing

Eriochrome cyanine R staining was used to stain oligodendrocytes, allowing for quantification of the white matter area ipsilateral and contralateral to injury. Both male and female SCI groups including groups treated with CBD exhibited SCI pathology unilaterally, with the side of injury exhibiting damage; additionally, LAM animals

exhibited no evidence of injury (Fig. 9 A). Upon quantification of white matter area, it was observed that the area contralateral to SCI remained statistically similar to surgical controls in both sexes (Fig. 9 C). It was further seen that SCI significantly decreased white matter ipsilateral to injury (Fig. 9 B). In addition, sex differences were observed between male and female SCI Veh animals, where female SCI Veh animals had significantly greater white matter area ipsilateral compared to their male counterparts (Fig. 9 C). Drug effects were also observed in females, CBD treated females had significantly less white matter compared to non-treated SCI females. There was no drug effect observed in male SCI animals.

Discussion

The purpose of this study was to evaluate the effects of acute CBD administration on outcomes post-SCI chronically in both males and females. It was found that SCI significantly diminished paw function and decreased neuronal numbers and white matter area ipsilateral to injury in both sexes. Behaviorally, it was shown that SCI induced increased incidence of contralateral hind paw withdrawal to cold stimuli but not mechanical force in males and females. Utilizing the grimace assessment, it was seen that all female SCI groups exhibited increased facial grimace scores after acetone application and compared to control groups, and male SCI CBD also exhibited increased facial scores post acetone application and male SCI Veh animals trended on this. In regards to sex effects, it was shown that female animals had greater white matter area preservation post-SCI compared to their male counterparts. Additionally, it was observed that CBD

treatment in males prevented diminished gains in body mass post-SCI, as observed in the SCI non-treated animals. CBD treatment had intermittent effects in males, but no lasting chronic effects, whereas trends of potential chronic protection was observed in females treated with CBD.

As observed in past studies, the model utilized in this study induced chronic issues in both sexes such as diminished functional capacity, allodynia, neuronal and white matter damage which are observed clinically [5-7, 53, 54]. The dose of CBD used in this study resulted in plasma levels similar to what has been observed in past studies [55-57]. Both untreated and treated SCI groups exhibited behavioral changes post injury, however males and females did not significantly differ from each other indicating that SCI affected both sexes. Histologically, all SCI groups exhibited neuronal loss and white matter damage. Though no behavioral differences existed between males and females, untreated SCI females had greater white matter area compared to their male counterparts. Previous studies have shown that females have greater protection post SCI compared to males. Specifically, estrogen (E) and E related compounds aid in imparting protection post injury through reducing inflammation, production of oxidative compounds, glial cell activation, calpain and caspase activity [10, 11, 58-61].

Interestingly, CBD administration in females decreased white matter. SCI CBD females had white matter area similar to their male counterparts, and had no effect on male white matter area. A study investigating the effects of *C. sativa* constituents on E receptor binding found that *C. sativa* constituents, including CBD, decreased E2 binding [62]. As mentioned earlier, E imparts protection post SCI therefore inhibiting the ability for E binding acutely post injury may diminish the ability for E to exert protection, leading to

decreased white matter as observed in this study. Despite decreased white matter area in SCI CBD females, administration of drug inferred protection acutely post SCI from cold allodynia and maintained a trend of continued protection chronically. Neuronally, all SCI groups were statistically similar indicating that neuroprotective measures did not confer SCI-NP protection. Behaviorally, the neuronal and white matter damage resulted in diminished forepaw use which began acutely post injury and lasted chronically with no effect of CBD administration on motor function in both sexes. In future studies, the mechanism of this protective effect needs to be further elucidated by investigating the effects of CBD administration acutely post SCI on secondary injury as chronic observations did fully expound on this protective effect.

In the field of pain research, there has been great pressure and need to ensure that responses from animals are indeed pain responses [63-67]. In the field of SCI, this has great importance as spasticity is an outcome that can muddle whether or not withdrawal responses to stimuli are hyper-reflexive or pain [68-70]. To delineate between these responses in this study, a modified rat grimace scale was used [71, 72]. Using this assessment during the acetone test, it was shown that both female SCI groups and male SCI CBD had increased facial grimace scores compared to scores prior to acetone exposure, with male SCI Veh animals trending on increased facial grimace at Week 6 post injury, with no LAM animals exhibiting any increased facial grimace. Considering this, there is evidence to support the claim that the SCI model used results in the development of cold allodynic pain as observed in other SCI models and clinically [6, 7, 15, 16, 69, 73-75]. No drug effects nor sex differences were observed, indicating that CBD treatment did not affect quality of pain response and that both males and females

are susceptible to pain development post injury. Notably, during the behavioral assessment, SCI groups had greater paw withdrawal compared to their respective control groups indicating that parallel use of grimace during the acetone test yields results that support each other.

In addition to development of cold allodynia, animals that received SCI exhibited autophagic behavior in both sexes. This behavior was not displayed by any surgical control animal. In all animals that developed autophagia, the behavior was relegated to the hind paw contralateral to injury. In the field of SCI and pain, there are two sections of interest, at-level, and below-level pain development. Clinically, at-level pain can develop in patients and is commonly observed acutely post injury and tends to resolve within a month after SCI though some patients experience chronic at-level pain. Below-level pain can develop as well, usually several weeks to months after injury and typically does not resolve, resulting in chronic, neuropathic pain [76-80]. In models of neuropathic pain, autophagic behavior has been shown to be a phenotypic marker for neuropathic pain in rodent models of neuropathic pain including SCI models [18, 81-86]. SCI Veh animals had an incidence of 55% and 46% for males and females respectively. Administration of CBD reduced incidence of autophagic behavior in both sexes, 30% and 18% in males and females, lending further support that CBD confers protection against pain development.

Beyond the effects of CBD on histological and pain outcomes post SCI, CBD exhibited effects on body mass gains. It was shown that administration of CBD prevented diminished body mass gains post SCI in males compared to uninjured controls, which was seen in untreated SCI males. Beginning at Week 2 and lasting until the end of the study, SCI Veh males had significantly less body mass gains compared to LAM animals,

showing that SCI affects metabolism and hat CBD administration conferred protection against this. This effect was not observed in females. Clinically, acute weight loss has been observed post SCI as well [87]. Investigations into metabolic disturbances post SCI have determined that there is widespread, systemic inflammation that influence organ function, including those that affect metabolic function such as the pancreas and intestinal system [87-92]. The pancreas has been shown to undergo inflammasome production as well as increased caspase cleavage leading to organ damage and metabolic disorders chronically post injury [92]. Within the intestinal system, SCI was shown to decrease blood flow to the superior mesenteric artery (SMA), which supports blood flow to the entire intestinal system. Additionally, it was shown that introduction of nutrients to the intestinal system did not result in immediate increase in blood flow to the intestinal system, as blood flow through the SMA was halted or greatly delayed post SCI with these issues resolving 3 weeks after injury in rats [90].

Regarding the effects of CBD on the intestinal system, several studies have shown that CBD has protective effects on the gastrointestinal system [38, 93-96]. A study investigating the effects of the neuroimmune axis on gut inflammation found that CBD administration decreased inflammation via activation of peroxisome proliferator-activated receptor gamma (PPARγ), a complex of proteins that affect transcription [38]. Beyond this, a study examining the effects of CBD on an animal model of colitis found that CBD treatment decreased intestinal damage and neutrophil related inflammation [97]. Though this study did not further investigate the protective effect of CBD on body mass post injury, it is something that merits further examination as metabolic dysfunction post SCI is a chronic issue post injury.

In conclusion, acute administration of CBD post SCI conferred some chronic protection in both male and female animals. In regards to males, SCI animals treated with CBD did not experience diminished gains in mass. In regards to females, CBD treatment protected SCI animals acutely from cold allodynia and trended on chronic protection. Both sexes had decreased incidence of autophagic behavior in CBD treated animals. When tissue was analyzed for possible mechanism based on white matter sparing or neuroprotective effects, it was shown that CBD protection did not exert neuroprotective effects and decreased white matter volume in females. Future studies investigating possible mechanism of action should consider looking at acute and sub-acute timeframes as well as intermediate components of 2° injury and the effect of CBD on these factors. In addition to mechanism determination, it would behoove future studies investigating therapeutic potential of CBD on SCI-NP to examine prolonged treatment schedule.

Acknowledgements

Thanks to members of the Floyd and McAllister labs who aided in the execution of this project, especially Tracy Niedzelko, Sabrina Davis, Hamelmal Kassahun, and Maddy Gohlke. Additional thanks to funding sources: Conquer Paralysis Now Grant (CF) and the TJ Atchinson SCI Research Program at UAB and Conquer Paralysis Now Grant (CF, SM).

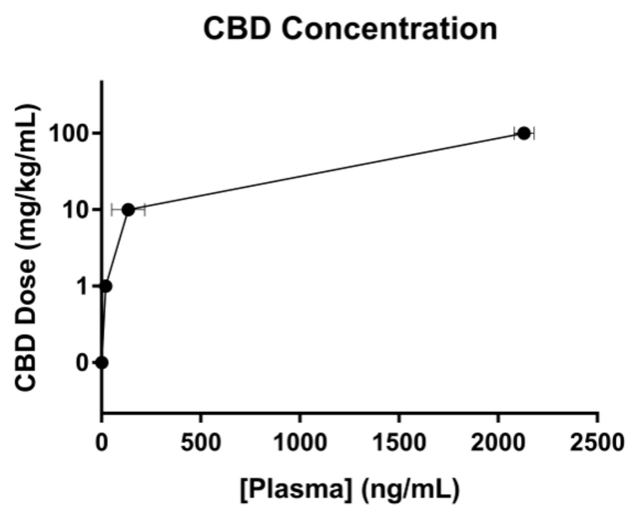


Figure 1: Cannabidiol plasma concentrations. One hour post i.p. injection cannabidiol (CBD) was detectable within rodent plasma. CBD was given in a logarithmic fashion (0, 1, 10, and 100 mg/kg/mL), with plasma CBD concentration increasing with each dose. Data shown are means +/- s.e.m., n = 2 per group.

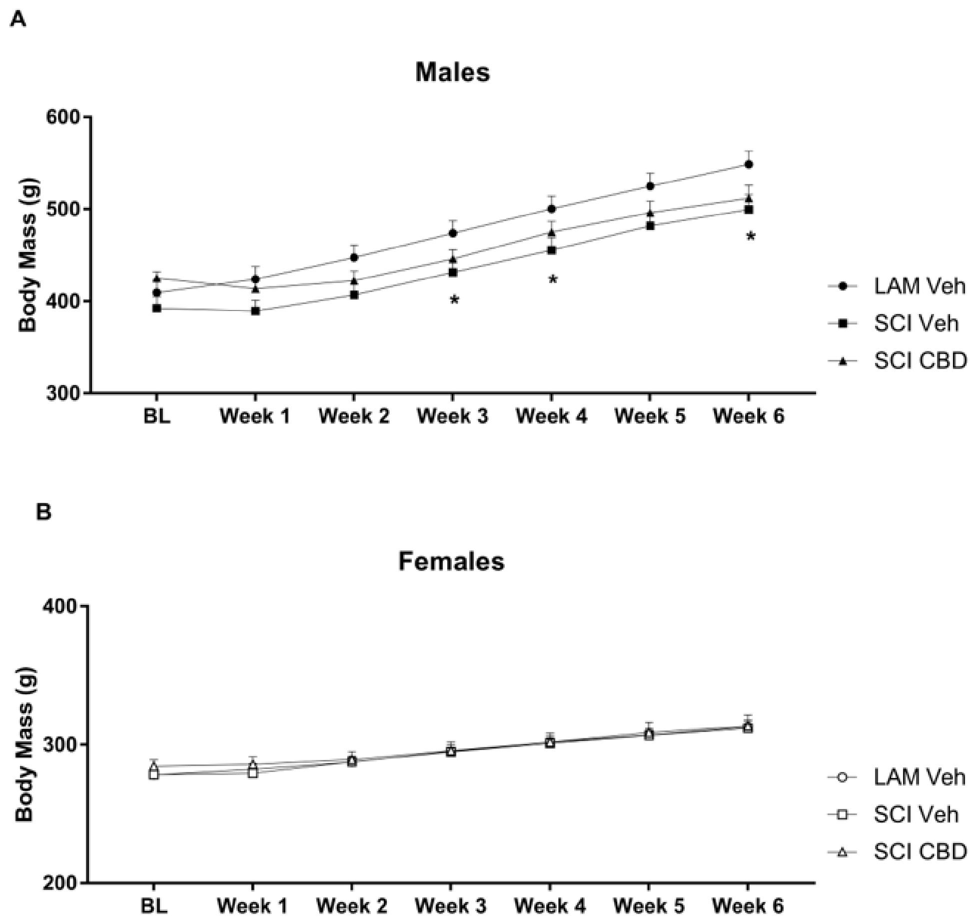


Figure 2: Body mass gains are affected by injury and drug treatment in males. Animals were weighed daily prior to and after surgery. Male spinal cord injured (SCI) untreated (vehicle; Veh) animals had diminished gains in mass (grams; g) at Weeks 3, 4, and 6 post injury compared to surgical controls (laminectomy; LAM). Male SCI animals treated with cannabidiol (CBD) had no diminished gains. Female SCI animals had no difference in mass gains post injury. Data shown are means +/- s.e.m., n = 10-13 per group, and analyzed via 2-way ANOVA, *: $p \leq 0.05$.

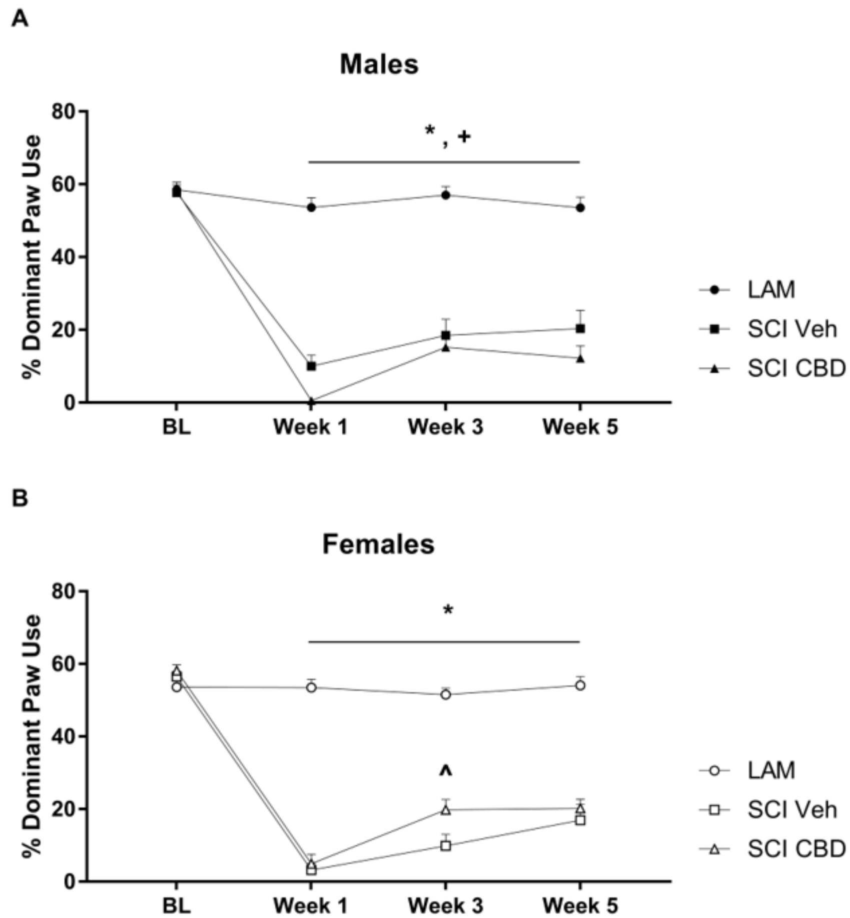


Figure 3: Motor function is affected by injury with intermittent effects of drug treatment and not affected by sex. Animals were assessed prior to, then biweekly post-surgery on motor function via paw placement. Both groups of spinal cord injured (SCI) animals displayed significantly less motor function in the affected limb throughout the duration of the study compared to surgical controls (laminectomy; LAM) in males and females. There was no effect of cannabidiol (CBD) or sex. Data shown are means +/- s.e.m., n = 10-13 per group, and analyzed via 2-way ANOVA, *, ^: $p \leq 0.05$.

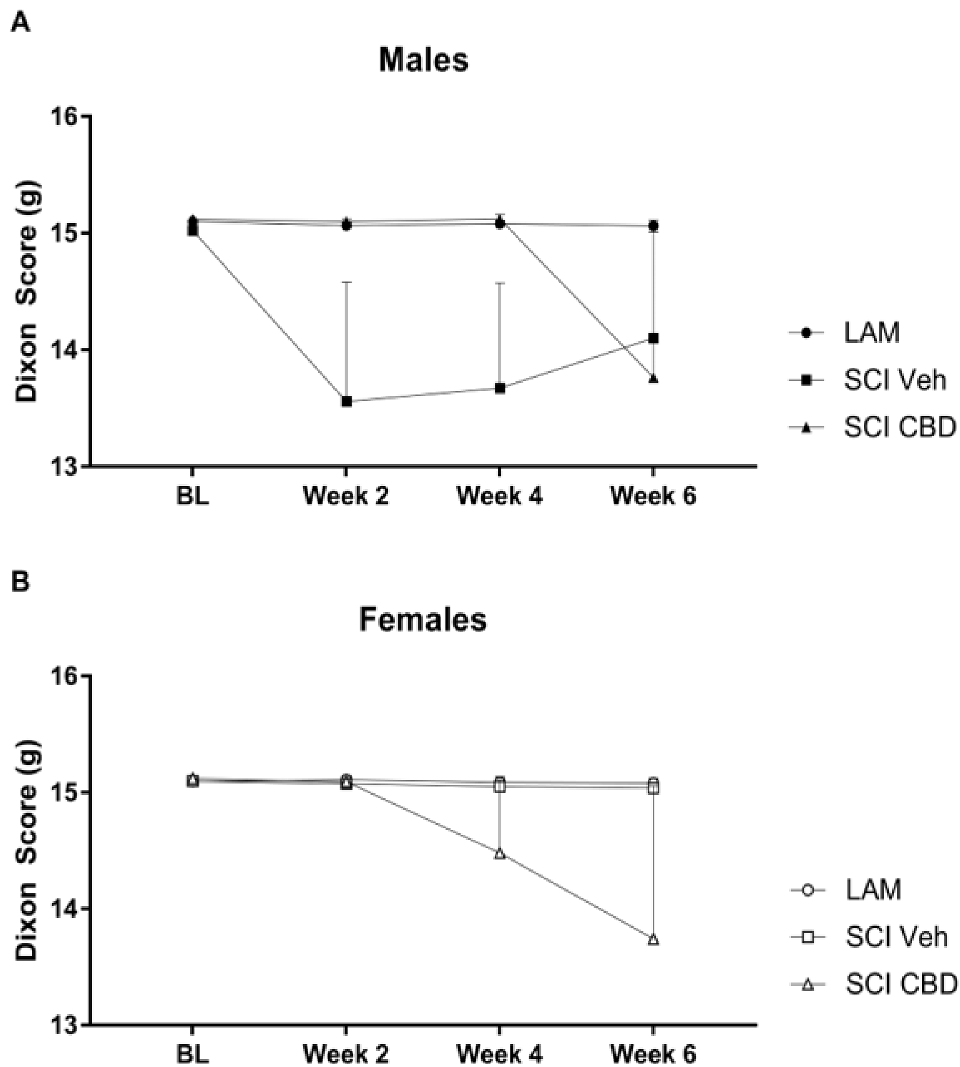


Figure 4: Mechanical sensitivity is not affected by injury, drug treatment, or sex. Before and after surgery, animals were assessed on mechanical sensitivity via Von Frey using the up-down method. There was no effect of sex, injury (spinal cord injury; SCI), or drug treatment (cannabidiol, CBD). Data shown are means +/- s.e.m., n = 10-13 per group, and analyzed via 2-way ANOVA.

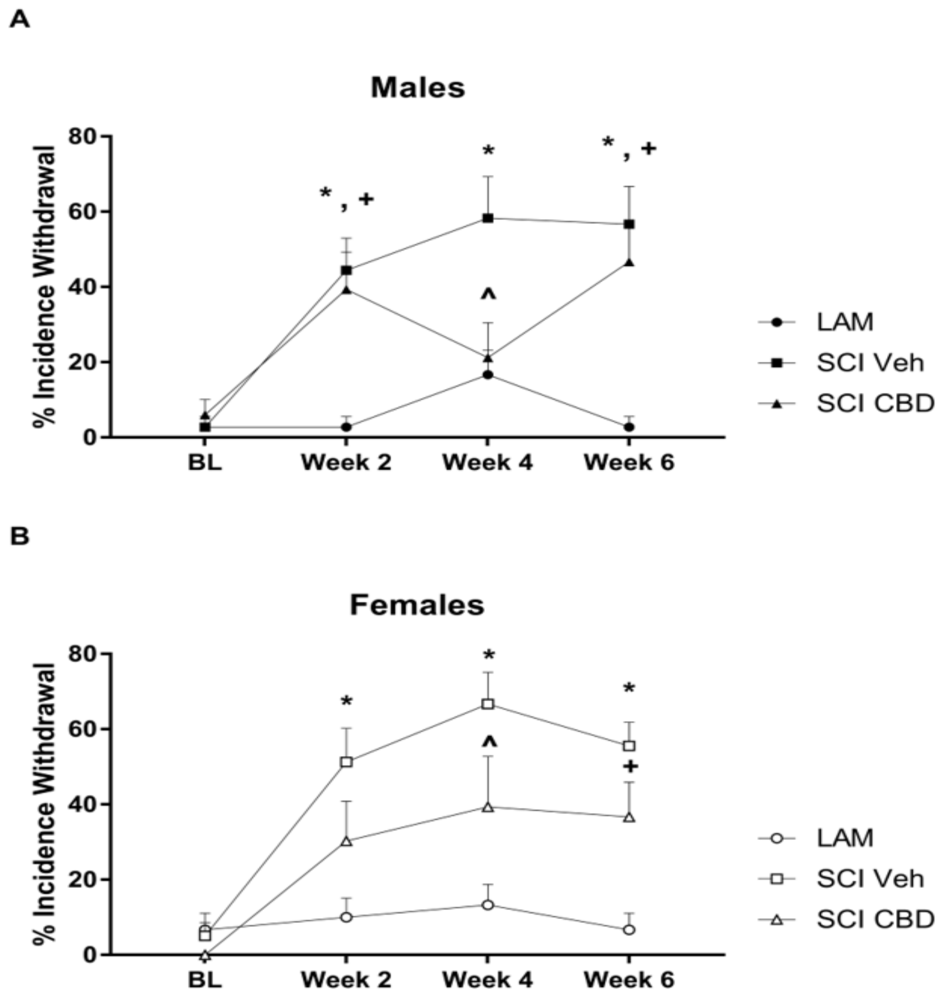


Figure 5: Cold sensitivity if affected by injury and drug treatment in both sexes. Using the acetone test, animals were assessed on cold sensitivity prior to and after surgery. Male and female untreated (vehicle, Veh) spinal cord injured (SCI) animals exhibited increased incidence of paw withdrawal compared to uninjured controls (laminectomy; LAM). At Week 2, female SCI CBD animals trended on diminished incidence of withdrawal ($p = 0.1123$) compared to female SCI Veh animals. At Week 4 cannabidiol (CBD) treatment decreased incidence of withdrawal in both sexes. In males, the effect was no longer present at Week 6, in females the effect was no longer presented though trended on

significance (p = 0.1953). Data shown are means +/- s.e.m., n = 10-13 per group, and analyzed via 2-way ANOVA, *,+,^: $p \leq 0.05$.

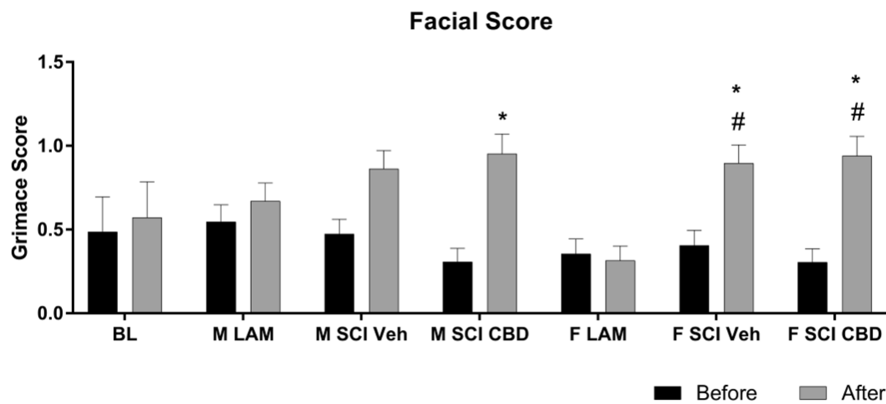


Figure 6: Facial grimace scores are affected by injury and application of cold stimuli in both sexes. During the acetone test, animals were video recorded and the resultant stills were used to assess facial score using the grimace assessment. Uninjured controls (laminectomy; LAM) had facial scores similar to baseline (BL) values. Facial scores prior to acetone application at Week 6 in spinal cord injured (SCI) animals did not differ from LAM animals nor their respective control groups in regards to sex (male; M and female; F) or drug treatment (control; vehicle; Veh and cannabidiol; CBD). Post acetone application, F SCI Veh, F SCI CBD, and M SCI CBD animal exhibited increased grimace facial scores with M SCI Veh trending on significance (p = 0.1106). Data shown are means +/- s.e.m., n = 10-11 per group, and analyzed via 2-way ANOVA, *, #: $p \leq 0.05$.

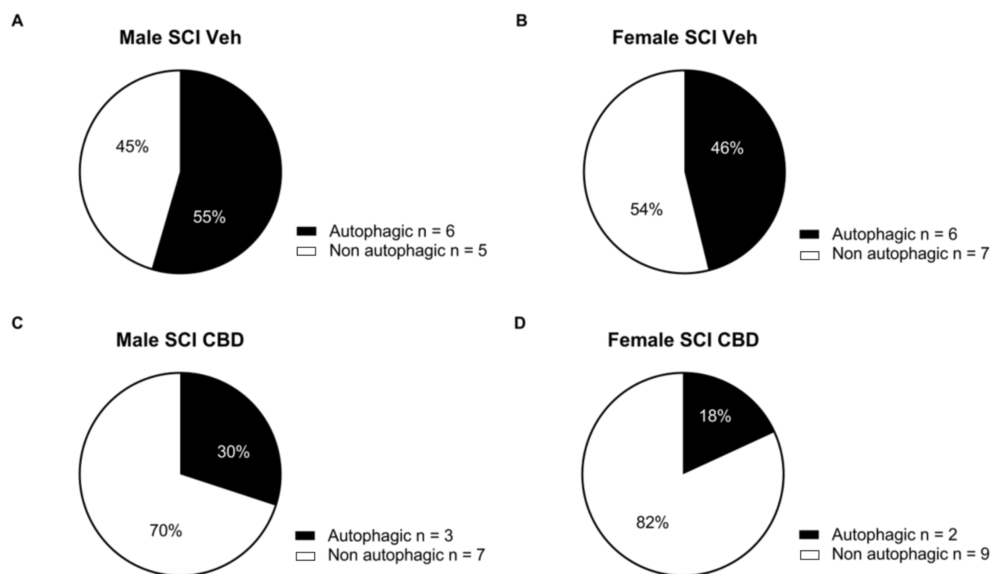


Figure 7: Injury induces autophagic behavior in both sexes and is affected by drug treatment. Animals were observed daily post surgery and observed for autophagic behavior. No surgical controls exhibited autophagia. All spinal cord injured (SCI) groups exhibited incidence of autophagia, 55% and 46% respectively for male and female SCI vehicle (Veh) treated animals and 30% and 18% for male and female SCI cannabidiol (CBD) treated animals. Data shown is percent incidence of autophagia, n = 10-13 per group.

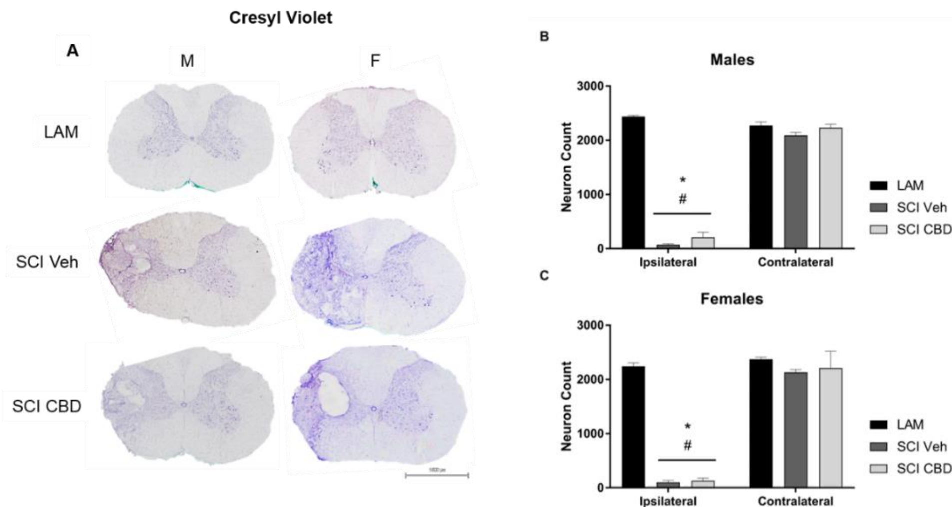


Figure 8: Injury diminishes neuronal numbers with no effect of sex or drug treatment. At Week 6, animals were euthanized and cervical tissue (vertebral level 5, C5) was collected, sliced, and stained with cresyl violet with spinal cord injury (SCI) pathology presenting unilaterally in both male (M) and female (F) SCI groups. No pathology was present in surgical controls (laminectomy, LAM). When quantified, the epicenter of injury exhibited decreased neuronal numbers compared to both LAM and the area contralateral to SCI. Data shown are means +/- s.e.m., n = 10-13 per group, and analyzed via 2-way ANOVA, *, #: $p \leq 0.05$.

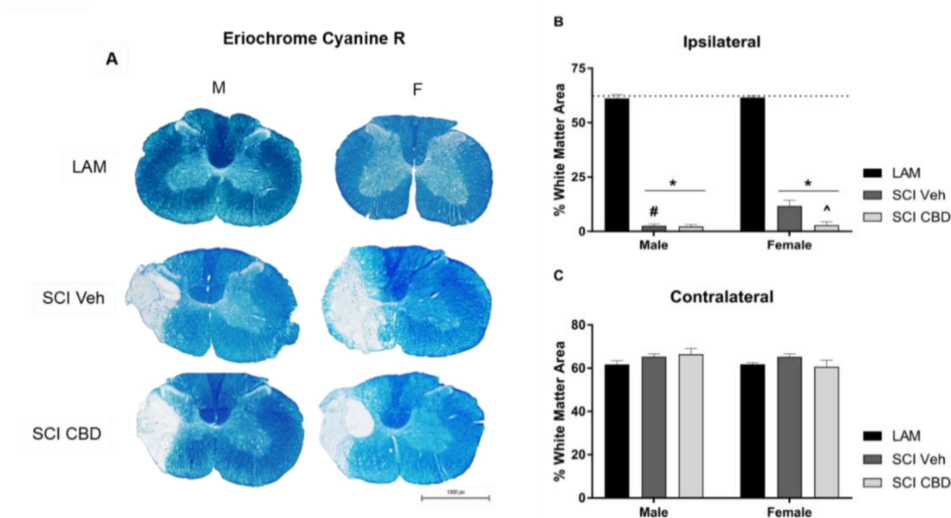


Figure 9: White matter area is negatively impacts by injury, with sex differences and drug treatment effects observed. At the end of the study, cervical tissue (vertebral level 5, C5) was collected, sliced, and stained with eriochrome cyanine R with spinal cord injury (SCI) pathology presenting unilaterally in both male (M) and female (F) SCI groups. No pathology was present in surgical controls (laminectomy, LAM). White matter area was quantified at the SCI epicenter. The area contralateral to injury (dashed line) was similar amongst all groups, with the area ipsilateral to injury to exhibiting decreased myelin area. F SCI vehicle (Veh) animals had greater white matter area compared to M SCI Veh and F SCI cannabidiol (CBD) treated animals. Data shown are means +/- s.e.m., n = 10-13 per group, and analyzed via 2-way ANOVA, *, #: $p \leq 0.05$.

References

1. Center, T.N.S.S., *Spinal Cord Injury Fact and Figures at a Glance*, U.o.A.a. Birmingham, Editor. 2016, University of Alabama at Birmingham: Birmingham, AL.
2. Saulino, M., *Spinal cord injury pain.* Phys Med Rehabil Clin N Am, 2014. 25(2): p. 397-410.
3. Gureje, O., et al., *Persistent pain and well-being: a World Health Organization Study in Primary Care.* Jama, 1998. 280(2): p. 147-51.
4. Hammell, K.R., *Spinal cord injury rehabilitation research: patient priorities, current deficiencies and potential directions.* Disabil Rehabil, 2010. 32(14): p. 1209-18.
5. Ataoglu, E., et al., *Effects of chronic pain on quality of life and depression in patients with spinal cord injury.* Spinal Cord, 2013. 51(1): p. 23-6.
6. Bouhassira, D., et al., *Prevalence of chronic pain with neuropathic characteristics in the general population.* Pain, 2008. 136(3): p. 380-7.
7. Anke, A.G., A.E. Stenehjem, and J.K. Stanghelle, *Pain and life quality within 2 years of spinal cord injury.* Paraplegia, 1995. 33(10): p. 555-9.
8. Felix, E.R., Y. Cruz-Almeida, and E.G. Widerström-Noga, *Chronic pain after spinal cord injury: What characteristics make some pains more disturbing than others?* The Journal of Rehabilitation Research and Development, 2007. 44(5): p. 703.
9. Sorge, R.E., et al., *Different immune cells mediate mechanical pain hypersensitivity in male and female mice.* Nat Neurosci, 2015. 18(8): p. 1081-3.
10. Chaovipoch, P., et al., *17beta-estradiol is protective in spinal cord injury in post- and pre-menopausal rats.* J Neurotrauma, 2006. 23(6): p. 830-52.
11. Kachadroka, S., et al., *Effect of endogenous androgens on 17beta-estradiol-mediated protection after spinal cord injury in male rats.* J Neurotrauma, 2010. 27(3): p. 611-26.
12. Mouton, P.R., et al., *Age and gender effects on microglia and astrocyte numbers in brains of mice.* Brain Res, 2002. 956(1): p. 30-5.
13. Detloff, M.R., R.E. Wade, Jr., and J.D. Houle, *Chronic at- and below-level pain after moderate unilateral cervical spinal cord contusion in rats.* J Neurotrauma, 2013. 30(10): p. 884-90.
14. Yezierski, R.P., et al., *Excitotoxic spinal cord injury: behavioral and morphological characteristics of a central pain model.* Pain, 1998. 75(1): p. 141-55.
15. Bouhassira, D., et al., *Comparison of pain syndromes associated with nervous or somatic lesions and development of a new neuropathic pain diagnostic questionnaire (DN4).* Pain, 2005. 114(1-2): p. 29-36.
16. Dworkin, R.H., et al., *Symptom profiles differ in patients with neuropathic versus non-neuropathic pain.* J Pain, 2007. 8(2): p. 118-26.
17. Costigan, M., J. Scholz, and C.J. Woolf, *Neuropathic pain: a maladaptive response of the nervous system to damage.* Annu Rev Neurosci, 2009. 32: p. 1-32.
18. Xu, X.J., et al., *Chronic pain-related syndrome in rats after ischemic spinal cord lesion: a possible animal model for pain in patients with spinal cord injury.* Pain, 1992. 48(2): p. 279-90.
19. EUGENE PARK ALEXANDER A. VELUMIAN, a.M.G.F., *The Role of Excitotoxicity in Secondary Mechanisms of Spinal Cord Injury: A Review with an*

Emphasis on the Implications for White Matter Degeneration. Journal of Neurotrauma, 2004. 21(6): p. 21.
20. Hausmann, O.N., *Post-traumatic inflammation following spinal cord injury.* Spinal Cord, 2003. 41(7): p. 369-78.
21. Khan, T., et al., *Animal models of spinal cord contusion injuries.* Lab Anim Sci, 1999. 49(2): p. 161-72.
22. Oyinbo, C.A., *Secondary injury mechanisms in traumatic spinal cord injury:a nugget of this multiply cascade.* Acta Neurobiologiae Experimentalis, 2011. 71: p. 19.
23. Sekhon, L.H. and M.G. Fehlings, *Epidemiology, demographics, and pathophysiology of acute spinal cord injury.* Spine (Phila Pa 1976), 2001. 26(24 Suppl): p. S2-12.
24. Esposito, E. and S. Cuzzocrea, *Anti-TNF therapy in the injured spinal cord.* Trends Pharmacol Sci, 2011. 32(2): p. 107-15.
25. Gorio, A., et al., *Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma.* Proc Natl Acad Sci U S A, 2002. 99(14): p. 9450-5.
26. Xu, J., et al., *Methylprednisolone inhibition of TNF-alpha expression and NF-kB activation after spinal cord injury in rats.* Brain Res Mol Brain Res, 1998. 59(2): p. 135-42.
27. Breivik, H., et al., *Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment.* Eur J Pain, 2006. 10(4): p. 287-333.
28. Johnson, J.R., et al., *Multicenter, double-blind, randomized, placebo-controlled, parallel-group study of the efficacy, safety, and tolerability of THC:CBD extract and THC extract in patients with intractable cancer-related pain.* J Pain Symptom Manage, 2010. 39(2): p. 167-79.
29. Wade, D.T., et al., *A preliminary controlled study to determine whether whole-plant cannabis extracts can improve intractable neurogenic symptoms.* Clin Rehabil, 2003. 17(1): p. 21-9.
30. Weber, J., et al., *Tetrahydrocannabinol (Delta 9-THC) Treatment in Chronic Central Neuropathic Pain and Fibromyalgia Patients: Results of a Multicenter Survey.* Anesthesiol Res Pract, 2009. 2009.
31. Ward, S.J., et al., *Cannabidiol inhibits paclitaxel-induced neuropathic pain through 5-HT(1A) receptors without diminishing nervous system function or chemotherapy efficacy.* Br J Pharmacol, 2014. 171(3): p. 636-45.
32. Agurell, S., et al., *Pharmacokinetics and metabolism of delta 1-tetrahydrocannabinol and other cannabinoids with emphasis on man.* Pharmacol Rev, 1986. 38(1): p. 21-43.
33. Alozie, S.O., et al., *3H-delta 9-Tetrahydrocannabinol, 3H-cannabinol and 3H-cannabidiol: penetration and regional distribution in rat brain.* Pharmacol Biochem Behav, 1980. 12(2): p. 217-21.
34. Bergamaschi, M.M., et al., *Safety and side effects of cannabidiol, a Cannabis sativa constituent.* Curr Drug Saf, 2011. 6(4): p. 237-49.
35. Pertwee, R.G., *The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin.* Br J Pharmacol, 2008. 153(2): p. 199-215.

36. Bisogno, T., et al., *Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide.* Br J Pharmacol, 2001. 134(4): p. 845-52.
37. Costa, B., et al., *The non-psychoactive cannabis constituent cannabidiol is an orally effective therapeutic agent in rat chronic inflammatory and neuropathic pain.* Eur J Pharmacol, 2007. 556(1-3): p. 75-83.
38. De Filippis, D., et al., *Cannabidiol reduces intestinal inflammation through the control of neuroimmune axis.* PLoS One, 2011. 6(12): p. e28159.
39. Drysdale, A.J., et al., *Cannabidiol-induced intracellular Ca2+ elevations in hippocampal cells.* Neuropharmacology, 2006. 50(5): p. 621-31.
40. Esposito, G., et al., *Cannabidiol reduces Abeta-induced neuroinflammation and promotes hippocampal neurogenesis through PPARgamma involvement.* PLoS One, 2011. 6(12): p. e28668.
41. Hampson, A.J., et al., *Cannabidiol and (-)Delta9-tetrahydrocannabinol are neuroprotective antioxidants.* Proc Natl Acad Sci U S A, 1998. 95(14): p. 8268-73.
42. Harris, R.A. and J.A. Stokes, *Cannabinoids inhibit calcium uptake by brain synaptosomes.* J Neurosci, 1982. 2(4): p. 443-7.
43. Malfait, A.M., et al., *The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis.* Proc Natl Acad Sci U S A, 2000. 97(17): p. 9561-6.
44. Rajesh, M., et al., *Cannabidiol attenuates cardiac dysfunction, oxidative stress, fibrosis, and inflammatory and cell death signaling pathways in diabetic cardiomyopathy.* J Am Coll Cardiol, 2010. 56(25): p. 2115-25.
45. Ramer, R., et al., *COX-2 and PPAR-gamma confer cannabidiol-induced apoptosis of human lung cancer cells.* Mol Cancer Ther, 2013. 12(1): p. 69-82.
46. Ryan, D., et al., *Cannabidiol targets mitochondria to regulate intracellular Ca2+ levels.* J Neurosci, 2009. 29(7): p. 2053-63.
47. Vuolo, F., et al., *Evaluation of Serum Cytokines Levels and the Role of Cannabidiol Treatment in Animal Model of Asthma.* Mediators Inflamm, 2015. 2015: p. 538670.
48. Zuardi, A.W., *Cannabidiol: from an inactive cannabinoid to a drug with wide spectrum of action.* Revista Brasileira de Psiquiatria, 2008. 30(3): p. 10.
49. Bethea, J.R., et al., *Traumatic spinal cord injury induces nuclear factor-kappa B activation.* Journal of Neuroscience, 1998. 18(9): p. 3251-3260.
50. Charles H. tator, M.G.F., *Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms.* J Neurosurg, 1991. 75: p. 12.
51. Gilmer, L.K., et al., *Early mitochondrial dysfunction after cortical contusion injury.* J Neurotrauma, 2009. 26(8): p. 1271-80.
52. Shields, D.C., et al., *Calpain activity and expression increased in activated glial and inflammatory cells in penumbra of spinal cord injury lesion.* J Neurosci Res, 2000. 61(2): p. 146-50.
53. Smith, P.M. and N.D. Jeffery, *Histological and ultrastructural analysis of white matter damage after naturally-occurring spinal cord injury.* Brain Pathol, 2006. 16(2): p. 99-109.
54. Maynard, F.M., R.S. Karunas, and W.P. Waring, 3rd, *Epidemiology of spasticity following traumatic spinal cord injury.* Arch Phys Med Rehabil, 1990. 71(8): p. 566-9.

55. Grotenhermen, F., *Pharmacokinetics and Pharmacodynamics of Cannabinoids.* Clin Pharmacokinet, 2003. 42(4): p. 34.
56. Deiana, S., et al., *Plasma and brain pharmacokinetic profile of cannabidiol (CBD), cannabidivarine (CBDV), Delta(9)-tetrahydrocannabivarin (THCV) and cannabigerol (CBG) in rats and mice following oral and intraperitoneal administration and CBD action on obsessive-compulsive behaviour.* Psychopharmacology (Berl), 2012. 219(3): p. 859-73.
57. Hill, T.D., et al., *Cannabidivarin-rich cannabis extracts are anticonvulsant in mouse and rat via a CB1 receptor-independent mechanism.* Br J Pharmacol, 2013. 170(3): p. 679-92.
58. Samantaray, S., et al., *Low dose estrogen prevents neuronal degeneration and microglial reactivity in an acute model of spinal cord injury: effect of dosing, route of administration, and therapy delay.* Neurochem Res, 2011. 36(10): p. 1809-16.
59. Hauben, E., et al., *Sexual dimorphism in the spontaneous recovery from spinal cord injury: a gender gap in beneficial autoimmunity?* Eur J Neurosci, 2002. 16(9): p. 1731-40.
60. Sipski, M.L., et al., *Effects of gender on neurologic and functional recovery after spinal cord injury.* Arch Phys Med Rehabil, 2004. 85(11): p. 1826-36.
61. Sribnick, E.A., et al., *Estrogen attenuated markers of inflammation and decreased lesion volume in acute spinal cord injury in rats.* J Neurosci Res, 2005. 82(2): p. 283-93.
62. Sauer, M.A., et al., *Marijuana: interaction with the estrogen receptor.* J Pharmacol Exp Ther, 1983. 224(2): p. 404-7.
63. Vierck, C.J. and R.P. Yezierski, *Comparison of operant escape and reflex tests of nociceptive sensitivity.* Neuroscience & Biobehavioral Reviews, 2015. 51: p. 223-242.
64. Mogil, J.S., *Animal models of pain: progress and challenges.* Nat Rev Neurosci, 2009. 10.
65. Mogil, J.S. and S.E. Crager, *What should we be measuring in behavioral studies of chronic pain in animals?* Pain, 2004. 112.
66. Mogil, J.S., K.D. Davis, and S.W. Derbyshire, *The necessity of animal models in pain research.* Pain, 2010. 151.
67. Mogil, J.S., et al., *Hypolocomotion, asymmetrically directed behaviors (licking, lifting, flinching, and shaking) and dynamic weight bearing (gait) changes are not measures of neuropathic pain in mice.* Mol Pain, 2010. 6.
68. van Gorp, S., et al., *Translation of the rat thoracic contusion model; part 1-supraspinally versus spinally mediated pain-like responses and spasticity.* Spinal Cord, 2014. 52(7): p. 524-8.
69. Kramer, J.L.K., et al., *Neuropathic pain following traumatic spinal cord injury: Models, measurement, and mechanisms.* Journal of Neuroscience Research, 2017. 95(6): p. 1295-1306.
70. Yezierski, R.P., *Spinal cord injury pain: spinal and supraspinal mechanisms.* J Rehabil Res Dev, 2009. 46(1): p. 95-107.
71. Sotocinal, S.G., et al., *The Rat Grimace Scale: A partially automated method for quantifying pain in the laboratory rat via facial expressions.* Molecular Pain, 2011. 7(1): p. 55.
72. Schneider, L.E., et al., *Application of the Rat Grimace Scale as a Marker of Supraspinal Pain Sensation after Cervical Spinal Cord Injury.* J Neurotrauma, 2017.

73. Jung, J.I., et al., *Long-term Follow-up of Cutaneous Hypersensitivity in Rats with a Spinal Cord Contusion.* Korean J Physiol Pharmacol, 2008. 12(6): p. 299-306.
74. Dunham, K.A., et al., *Characterization of a graded cervical hemicontusion spinal cord injury model in adult male rats.* J Neurotrauma, 2010. 27(11): p. 2091-106.
75. Lindsey, A.E., et al., *An analysis of changes in sensory thresholds to mild tactile and cold stimuli after experimental spinal cord injury in the rat.* Neurorehabil Neural Repair, 2000. 14(4): p. 287-300.
76. Cardenas, D. and J. Rosenbluth, *At- and Below-Level Pain in Spinal Cord Injury: Mechanisms and Diagnosis*. Vol. 7. 2001. 30-40.
77. Hagen, E.M. and T. Rekand, *Management of Neuropathic Pain Associated with Spinal Cord Injury.* Pain and Therapy, 2015. 4(1): p. 51-65.
78. Werhagen, L., et al., *Neuropathic pain after traumatic spinal cord injury--relations to gender, spinal level, completeness, and age at the time of injury.* Spinal Cord, 2004. 42(12): p. 665-73.
79. Lee, S., et al., *Central Neuropathic Pain in Spinal Cord Injury.* Critical reviews in physical and rehabilitation medicine, 2013. 25(3-4): p. 159-172.
80. Siddall, P.J., et al., *A longitudinal study of the prevalence and characteristics of pain in the first 5 years following spinal cord injury.* Pain, 2003. 103(3): p. 249-257.
81. Xu, X.J., et al., *Up-regulation of cholecystokinin in primary sensory neurons is associated with morphine insensitivity in experimental neuropathic pain in the rat.* Neurosci Lett, 1993. 152(1-2): p. 129-32.
82. Neil, A., N. Attal, and G. Guilbaud, *Effects of guanethidine on sensitization to natural stimuli and self-mutilating behaviour in rats with a peripheral neuropathy.* Brain Res, 1991. 565(2): p. 237-46.
83. Seltzer, Z., et al., *Modulation of neuropathic pain behavior in rats by spinal disinhibition and NMDA receptor blockade of injury discharge.* Pain, 1991. 45(1): p. 69-75.
84. Dennis, S.G. and R. Melzack, *Self-mutilation after dorsal rhizotomy in rats: effects of prior pain and pattern of root lesions.* Exp Neurol, 1979. 65(2): p. 412-21.
85. Kauppila, T., *Correlation between autotomy-behavior and current theories of neuropathic pain.* Neuroscience & Biobehavioral Reviews, 1998. 23: p. 10.
86. Coderre, T.J., R.W. Grimes, and R. Melzack, *Deafferentation and chronic pain in animals: an evaluation of evidence suggesting autotomy is related to pain.* Pain, 1986. 26(1): p. 61-84.
87. Chen, Y., et al., *Obesity intervention in persons with spinal cord injury.* Spinal Cord, 2006. 44(2): p. 82-91.
88. Farkas, G.J. and D.R. Gater, *Neurogenic obesity and systemic inflammation following spinal cord injury: a review.* J Spinal Cord Med, 2017: p. 1-10.
89. Sun, X., et al., *Multiple organ dysfunction and systemic inflammation after spinal cord injury: a complex relationship.* J Neuroinflammation, 2016. 13(1): p. 260.
90. Besecker, E.M., et al., *Mesenteric vascular dysregulation and intestinal inflammation accompanies experimental spinal cord injury.* Am J Physiol Regul Integr Comp Physiol, 2017. 312(1): p. R146-r156.
91. Garshick, E., et al., *Systemic inflammation and reduced pulmonary function in chronic spinal cord injury.* Pm r, 2011. 3(5): p. 433-9.

92. Bigford, G.E., et al., *Neuroendocrine and cardiac metabolic dysfunction and NLRP3 inflammasome activation in adipose tissue and pancreas following chronic spinal cord injury in the mouse.* ASN Neuro, 2013. 5(4): p. 243-55.
93. Capasso, R., et al., *Cannabidiol, extracted from Cannabis sativa, selectively inhibits inflammatory hypermotility in mice.* British Journal of Pharmacology, 2008. 154(5): p. 1001-1008.
94. Esposito, G., et al., *Cannabidiol in Inflammatory Bowel Diseases: A Brief Overview.* Phytotherapy Research, 2013. 27(5): p. 633-636.
95. Klein, T.W., *Cannabinoid-based drugs as anti-inflammatory therapeutics.* Nat Rev Immunol, 2005. 5(5): p. 400-11.
96. Alhouayek, M. and G.G. Muccioli, *The endocannabinoid system in inflammatory bowel diseases: from pathophysiology to therapeutic opportunity.* Trends Mol Med, 2012. 18(10): p. 615-25.
97. Jamontt, J.M., et al., *The effects of Delta-tetrahydrocannabinol and cannabidiol alone and in combination on damage, inflammation and in vitro motility disturbances in rat colitis.* Br J Pharmacol, 2010. 160(3): p. 712-23.

DISCUSSION

Overarching Goals

The goals of this dissertation work was to 1) better understand what contributes to SCI-NP development and 2) investigate the therapeutic potential of CBD in protecting against SCI-NP development. The stress study presented here aided in characterizing the effects of chronic stress on SCI-NP post injury, which were previously unknown. It was shown that chronic stress exposure increased cold allodynic responses in animals with greater basal CORT release. The second study examined the effects of acute CBD treatment on the chronic outcome of SCI-NP. Through this study, it was shown that acute CBD treatment imparted protective effects against SCI-NP development in both males and females, with trends of chronic protection in females. These studies both filled gaps in knowledge within the SCI field, and provide a platform for further investigation.

Major Findings

SCI Model

The studies conducted in this dissertation utilized an animal model of SCI, incomplete tetraplegia, which exhibits a high degree of clinical relevance [1]. Similar to what has

been observed in the literature and clinically, this model results in motor function loss, sensory dysfunction, and development of NP [2-4]. In both studies, motor dysfunction, measured by paw placement, was observed acutely after injury, day 1, and persisted chronically up to the end of both studies, days 30 and 42 in the stress and CBD study respectively. Beyond motor dysfunction, sensory changes were also observed. SD animals that received a SCI developed cold allodynia, with symptoms being observed several weeks post injury and plateauing at xx week. Histologically, the model utilized also exhibited neuronal loss and myelin damage both of which are hallmarks of SCI [2, 5-8].

Additionally, the injury model of SCI produced NP with detectable symptoms beginning sub-acutely at approximately 2 weeks and persisting chronically, until the end of both studies weeks 4 and 6 for the stress and CBD study respectively. Within the clinical SCI population, NP typically develops weeks to months post injury and persists chronically demonstrating the translational relevance of this model [9-13]. In the field of SCI research, there has been a growing need to distinguish between hyper-reflexive spastic withdrawal and evoked pain withdrawal from stimuli, as spasticity is a common issue post injury in both humans and animal models of SCI [14-20]. Considering the communication gap between humans and rodents, the ability to distinguish pain from non-pain responses must rely heavily on non-verbal mechanisms.

Research investigating supraspinal mechanisms of pain determined that rodents produce facial grimaces when in pain, additionally these facial grimaces have been evaluated in an animal model of SCI and further characterized by our laboratory [3, 21-23]. The studies conducted mirrored clinical efforts to determine pain responses in non-verbal

patients such as comatose and intubated individuals and children [24-26]. Through these clinical studies, it was demonstrated that pain was associated with specific facial grimaces including orbital, nose, and mouth changes. These facial actions were also recapitulated in the animal studies leading to the development of the SCI grimace scale [3].

The CBD study utilized the modified grimace scale. It was found that surgical controls had no change in facial score compared to baseline and after acetone application. Additionally, both female SCI groups, and SCI males who received CBD had increased facial grimace scores and untreated SCI males trending on greater scores post acetone application. Overall, these findings support the use of grimace to assess supraspinal mechanisms of SCI-NP in assessing evoked pain as increased grimace scores, and trends of increased score, were observed post acetone application.

Beyond facial score, in the CBD study, it was observed that a portion of animals within the SCI groups developed autophagic behavior. Autophagic behavior was classified as chewing or licking of the hind paw for three consecutive days or longer without cessation. This behavior was only observed in animals that received an SCI, and solely occurred in the hind paw contralateral to injury. The importance of this two-fold. First, in the clinical population, SCI-NP is experienced below lesion where the source of pain is due to abnormal nerve signaling in the CNS and not damage to the peripheral nervous system – the defining characteristic of NP [27-29]. Second, the area of chewing/ licking solely occurred contralateral to injury. This is highly important, as pain processing pathways decussate at the spinal level, meaning that below level sensory dysfunction will occur contralateral to CNS injury, whereas motor function will be ipsilateral to

injury as this tract crosses at the brain level [30, 31]. Other studies examining NP have used autophagic behavior as an indicator of pain development, supporting using this behavioral phenotype to distinguish animals that have developed NP from animals that have not [32-34].

Approximately half of untreated SCI groups, males and females exhibited this behavior. This variability mimics what is observed in the human population, as it has reported that anywhere from 60 – 80% of SCI patients go on to develop NP [10, 12, 35, 36]. The stress study performed helps to provide some answers as to why this variability exists, as this study displayed that pre-injury experiences can influence SCI-NP outcomes. Other studies examining the role of stress and pain have found similar results, where exposure to stress prior to injury resulted in greater pain incidence [37-41]. This has also been observed in the clinical SCI population, where veterans who were exposed to stress prior to SCI, and subsequently developed PTSD, had greater quality of pain compared to SCI patients without PTSD [42].

In totality, both studies provide strong evidence for the C5 incomplete SCI model for producing outcomes observed in the human population, namely motor deficits that begin acutely and last chronically, development of NP that begins sub-acutely and persists chronically, as well as histological changes like neuronal loss, and white matter damage. The uses of this model extend far beyond these studies, as it provides a strong model for investigating SCI.

CBD

One of the major findings from the studies conducted in this dissertation were the beneficial effects of CBD treatment post SCI. CBD was shown to impart protective effects against cold allodynia in both sexes with trends of continued protection in females. In males, CBD was shown to protect against diminished gains in mass. In both sexes, CBD decreased the incidence of autophagic behavior from approximately half to 30% and 18% in males and females, respectively. The data presented here demonstrate that administration of CBD post-injury diminished incidence of paw withdrawal, and therefore SCI-NP. A short coming of this study was that the mechanisms of CBD interaction on the acute stages of 2° injury, when the drug was administered, were not investigated. However, looking at the literature potential mechanisms for this protective effect can be hypothesized and used as fodder for future studies as managing chronic SCI-NP in the acute stages of SCI will allow the field to focus on a major patient need [12, 15, 35, 36, 43].

Potential targets can be determined by evaluating contributors to cold allodynia development after nerve injury. In a model of chemotherapy induced NP, it was found that animals that developed sensitivity to cold had increased expression of transient receptor potential ankyrin 1 (TRPA1), a channel that is permeable to calcium, sodium, and potassium ions, in the dorsal root ganglia where peripheral sensory information synapses with the CNS. Additionally, it was shown that overexpression of TRPA1 increased incidence of cold hyperalgesia [44, 45]. TRP expression has been shown to be affected by calcium presence, where greater calcium concentrations increases expression of this receptor [46, 47]. Furthermore, it has been shown that TRP receptor, including

TRPA1, overexpression contributes to central sensitization, a major component of SCI-NP [31, 48-51]. Considering this, a similar mechanism could be occurring with SCI induced NP as there is ionic dysregulation and an influx of calcium following injury.

CBD has been shown to exert effects on calcium in the CNS. Specifically, it has been shown that in non-excitable states CBD increased intracellular calcium, while in hyper-excitable environments with high potassium concentrations present, CBD decreased intracellular calcium uptake via interactions with the sodium exchanger [52, 53]. Considering this, it is plausible that in the acute stages of the 2° injury cascade that administration of CBD diminished intracellular calcium uptake, as these cells were in a hyper-excitable environment, which may have contributed to the trends of chronically diminished SCI-NP outcome in females as well as the decreased autophagia in both sexes. Not only is it important to study potential mechanisms of CBD and 2° injury, but it is also important to examine sex differences as sexually dimorphic effects were observed in the CBD study.

Sex Differences

Sex differences are important to study because within the chronic pain field, women make the vast majority of pain patients. The literature has shown that a mix of factors, sociocultural, mental, and biological factors, affect this skew [54-58]. Focusing on the biological contributions, E has been shown to influence pain. Specifically, it has been observed that stage of estrous cycle affects pain threshold, where low progesterone levels increase pain threshold, which has been found to continue until menopause thus

strengthening the claim that sex hormones play a large role in pain [56]. It has been shown that E affects synaptic plasticity, where exposure to E increases neuronal activity in the brain, and that women had greater brain activity in response to pain compared to males [55]. Beyond this, it was found that sex affects efficacy of pain therapeutics. A study investigating NP and the role of the immune system in male and female mice found that treatments targeting macrophages had greater analgesic effects in males compared to females, and that targeting T cells had greater therapeutic effect in females compared to males [59]. These studies highlight the importance of not only characterizing sex differences in pain disorders, but using both sexes to evaluate therapeutic efficacy, especially in the field of SCI-NP.

Though women make a large majority of pain patients, men make a greater proportion of SCI patients, nearly 80%. Interestingly, women who develop SCI-NP report greater quality of pain compared to their male counterparts, further emphasizing the need to evaluate both sexes [1, 35, 60]. In the CBD study conducted, it was observed that untreated SCI females had greater myelin preservation post injury compared to their male counterparts and had no incidence of diminished gains in mass post SCI as observed in males. In the literature and studies conducted in our laboratory, E has been shown to confer protection post SCI in females, supporting the findings of this study [61-63]. It has been shown that E reduces inflammation and apoptosis by reducing production of inflammatory cytokines via activation of the extracellular signal related kinases (ERK) which can induce transcription of anti-inflammatory compounds, additionally, E has been shown to prevent apoptosis by blocking caspase activation in the CNS [64, 65]. Further strengthening the assertion of E conferring protection post

SCI, E administration to males post SCI, provided protective effects through decreased inflammation, proteolytic activity, and apoptosis [65-70].

Interestingly, both males and females that received SCI developed NP and had similar incidence of hind paw withdrawal in response to acetone application, as well as development of autophagic behavior. Administration of CBD post-injury resulted in protection against NP development in the sub-acute time period in males and females while there were trends of chronic protection in females and not males; overall indicating CBD has potential for therapeutic quality at chronic time points in females. Additionally, CBD treatment significantly reduced autophagic behavior in both sexes.

Males and females underwent the same dosing protocol, an intraperitoneal injection of CBD 30 minutes after impact with one dose per day for seven consecutive days at a dose of 100 mg/kg/mL. This dosing regimen was shown to result in plasma CBD concentrations similar to literature values [71-73]. The purpose of this regimen was to target the acute phase of 2° injury, as 2° injury has been shown to begin within minutes after SCI [43, 74-76]. Though both sexes undergo 2° injury post injury, sex differences have been observed in regards to the acute phase of 2° injury. It has been shown that E protects against calcium loading, thereby protecting against the effects of calcium mediated apoptosis and glutamate mediated excitotoxicity [62]. These effects, coupled with the ability of CBD to protect against calcium mediated cell death and damage may be a potential explanation why CBD had greater protective effects in females.

As discussed earlier, these protective effects against NP and autophagic behavior may have been through interacting with components in the acute phase of 2° injury, such as

calcium dysregulation, which affect chronic outcomes such as receptor expression in the CNS. While not examined in the present dissertation, these changes have also been shown to exist in animals exposed to chronic stress, indicating that the pro-inflammatory, pro-oxidative environment induced by chronic stress prior to SCI could exacerbate acute components of 2° injury, leading to enhanced central sensitization which is a major contributor to NP development [28].

Chronic Stress

Exposure to chronic stress has been shown to contribute to central sensitization via alterations in neural circuitry [77-79]. Looking at the stress characterization study, SD animals exposed to chronic stress prior to SCI had increased incidence of withdrawal to cold stimuli compared to unstressed SCI groups, indicating that pre-injury stress events contributed to SCI-NP development. A shortcoming of this study was that the underlying mechanism for this effect was not determined. Looking at the literature, studies investigating chronic pain and the role of the HPA axis have found that HPA dysregulation aids in maintaining chronic pain conditions. Specifically, it has been found that changes in HPA responsiveness contribute to pain [80-83]. This phenomenon has specifically been demonstrated in animal models of chronic stress, where repeated stress exposure decreases CORT release and reduces HPA axis response to stress via CORT feeding back into the CNS system [84]. These changes have been shown to contribute to chronic pain clinically, where patients with HPA axis dysfunction exhibited increased incidence and severity of pain [85]. Considering the findings of the stress study and the literature support showing that chronic stress induces HPA axis

dysfunction, this is a possible explanation for how pre-injury stress events can affect chronic outcomes post SCI like SCI-NP.

As mentioned earlier, in the stress study conducted, SD animals had greater incidence of cold allodynic responses compared to LEW animals and non-stressed SD animals. In addition to this, it was found that SD animals had greater basal CORT release compared to LEW animals, which has also been reflected in the literature [86, 87]. Previous studies have also shown that greater exposure to CORT leads to the induction of a pro-inflammatory state. The mechanism of which is due to the increased production of pro-inflammatory cytokines caused by the inability of CORT to bind to the GC receptor. This is mediated by allosteric inhibition by FKBP 51, thereby disallowing for transcriptional changes that would normally induce transcription of anti-inflammatory cytokines [88-90]. Based on this, it can be postulated that the greater basal CORT observed in SD animals combined with chronic stress exposure induced a pro-inflammatory state, thereby exacerbating the inflammation observed during 2° injury thereby leading to increased severity of SCI-NP.

The results from this study shed light on how pre-injury events contribute to injury outcomes, specifically NP development. The contributions of 2° injury on SCI-NP development have been discussed in regards to CBD and stress study, with a possible mechanism discussed in regarding the CBD study. Looking at a potential mechanism in the stress study, based on the literature, it has been shown that exposure to chronic stress increases N-Methyl-D-Aspartate (NMDA) receptors thereby contributing to calcium dysregulation [91, 92]. NMDA receptors are activated by glutamate and upon activation an ion channel is opened allowing calcium to flow through [93]. Calcium ion

dysregulation is major hallmark of 2° injury. The ionic dysregulation begins acutely after SCI, as neurons undergo depolarization waves which allow calcium to enter the cell, contributing to cellular apoptosis, spreading the lesion area, and increasing TRP expression which has been shown to contribute to central sensitization and thus SCI-NP [28, 43, 46, 47, 51, 75, 94]. As mentioned earlier, a shortcoming of the stress study is that no mechanism for why chronic stress exposure increased SCI-NP development, however, looking at NMDA receptor expression, calcium dysregulation, and TRP expression could provide insight on this knowledge gap.

The results of the stress study conducted provide insight as to how pre-injury events can affect post injury outcomes. SCI is unique as the underlying cause is not primarily due to a disease or genetic cause, but due to accidental trauma [1]. As such, there is high variability between patients, which creates difficultly in predicting which patient will go on to develop SCI-NP, further confounded by SCI-NP development not being correlated to level or severity of injury [13, 36]. However, with this study in mind, it would be possible to determine patients at greater risk for SCI-NP development based on pre-injury stress load. Using this, caregivers could provide therapeutic interventions aimed at reducing 2° injury, which could prevent SCI-NP development.

Conclusion

Through these studies, it has been demonstrated that the SCI model utilized, C5 incomplete injury, exhibited high clinical relevance as evidenced by motor dysfunction, development of NP, neuronal loss, and myelin damage. Additionally, it was observed that events prior to SCI, specifically chronic stress, enhanced SCI-NP outcomes

chronically. Moreover, it was found that treatment with CBD acutely after SCI positively affected outcomes with sexually dimorphic effects as evidenced by cold allodynia protection sub-acutely in males and females and potentially chronically in females, and diminished autophagic behavior in both sexes. Together, these studies help shed insight on and offer an explanation for the high variability in patient outcomes, providing evidence that pre-injury factors and sex differences play a role in outcomes after SCI. The research conducted in this dissertation also demonstrate the potential of CBD as a promising therapeutic which should further be evaluated given the benefits observed at both acute and chronic time points in both sexes. In totality, these studies provide insight into contributors to and treatments for SCI-NP, while providing platforms for further investigation.

Future Studies

Both studies conducted were characterization studies, where the basic question whether an experimental variable would have an effect on outcomes post SCI. As such, the mechanism for how these experimental variables induced these outcomes remain largely unknown. Therefore, the obvious next steps with both studies is to determine the mechanism of action. Considering both studies had effects on SCI-NP, future studies should investigate how these variables affect factors known to contribute to SCI-NP development.

As discussed, 2° injury is a major contributor to SCI-NP and factors present during this cascade have been shown to be affected by both stress and CBD, namely calcium

dysregulation, glutamate mediated excitotoxicity, and TRP overexpression. Therefore, examining the effects of stress and CBD on 2° injury could elucidate how these variables affect SCI-NP development. The effects of 2° injury has potential to be examined at various stages post SCI, as 2° injury begins acutely and persists chronically. In the CBD study conducted, targeting the acute stage of 2° injury exerted protective effects, though with greater effects in females compared to males. This brings up the questions, what if CBD were given for a longer duration? Would the trends of chronic protection in females result in significant results and would longer CBD treatment impart continued protection in males chronically? Therefore, it would be of great importance to alter the timing, length of treatment, and dose of CBD in both sexes. Through this, it could be determined what, if any, therapeutic windows exist and if there are sex differences. Delving even deeper, it could be determined if sex differences exist in regards to what aspects of 2° injury are being affected by CBD.

As discussed earlier, the 2° injury is a multifaceted cascade that changes temporally. Future investigations into factors affected by pre-injury stress and CBD, though unstudied, can be based on looking at previous studies which have been discussed earlier. Broken down temporally, in the acute stages of 2° injury calcium dysregulation and glutamate mediated excitotoxity, in the sub-acute stages, immune cell recruitment and infiltration, and receptor expression in the chronic stages, particularly NMDA and TRP expression would be candidates of interest. The factors listed have also been implicated contributing to central sensitization and the development of SCI-NP as discussed earlier.

From these experiments, one could perform a series of mimic/block experiments on one facet of 2° injury and determine the subsequent effect on central sensitization as well as SCI-NP. For example, to examine the role of glutamate mediated excitotoxity and its role in central sensitization, an NMDA antagonist could be used to block the receptor and an agonist could be used to activate the receptor after injury. Using this set of experiments, the incidence of SCI-NP could be determined using behavioral assessments such as the ones used in the studies in this dissertation. In addition to behaviors, CNS tissue could be examined for receptor changes, such as TRP, to determine any chronic changes. Taken together, these experiments would provide a mechanism for how pre-injury events contribute to SCI-NP, how CBD interacts with 2° injury, how much these factors contribute to SCI-NP, and if sexually dimorphic effects exist thereby leading to determination of therapeutic windows for each sex.

Further research into these topics would ultimately benefit the human SCI population. The first benefit would be greater insight into 2° injury, which as discussed, is a large contributor to the spread of damage post injury and to central sensitization. Advanced knowledge of these aspects would benefit the field of SCI and provide knowledge for supporting investigations into other key patient needs. Secondly, the process of how pre-injury events contribute to factors post injury would be elucidated.

Using these results, the ability to incorporate this knowledge into treatments acutely after SCI could aid in the prevention of SCI-NP development. More specifically, pre-injury events could aid in determining patients at increased risk for SCI-NP development, hence indicating the need for therapeutic intervention against 2° injury. Thirdly, the specific interaction of CBD with 2° injury and temporal window can be

determined by additional research, information which would help to determine timing and length of treatment in the patient population, which may differ based on sex.

Ultimately, further examination of the studies performed would contribute valuable knowledge to the SCI field, leading to improved acute care for SCI patients. Enhanced acute care would result in improved chronic outcomes, specifically regarding SCI-NP, which is a top patient concern. Most importantly, improving SCI-NP treatment and management will lead to a better patient quality of life.

LIST OF REFERENCES

1. Singh, A., et al., *Global prevalence and incidence of traumatic spinal cord injury.* Clin Epidemiol, 2014. **6**: p. 309-31.

2. Center, T.N.S.S., *Spinal Cord Injury Fact and Figures at a Glance*, U.o.A.a. Birmingham, Editor. 2016, University of Alabama at Birmingham: Birmingham, AL.

3. Nógrádi, A. and G. Vrbová, *Anatomy and physiology of the spinal cord*, in *Transplantation of Neural Tissue into the Spinal Cord*. 2006, Springer. p. 1-23.

4. Panjabi, M.M., et al., *Multidirectional instabilities of traumatic cervical spine injuries in a porcine model.* Spine (Phila Pa 1976), 1989. **14**(10): p. 1111-5.

5. Southern, E.P., et al., *Cervical spine injury patterns in three modes of high-speed trauma: a biomechanical porcine model.* J Spinal Disord, 1990. **3**(4): p. 316-28.

6. Marcon, R.M., et al., *Fractures of the cervical spine.* Clinics (Sao Paulo), 2013. **68**(11): p. 1455-61.

7. Anke, A.G., A.E. Stenehjem, and J.K. Stanghelle, *Pain and life quality within 2 years of spinal cord injury.* Paraplegia, 1995. **33**(10): p. 555-9.

8. Ataoglu, E., et al., *Effects of chronic pain on quality of life and depression in patients with spinal cord injury.* Spinal Cord, 2013. **51**(1): p. 23-6.

9. Hammell, K.R., *Spinal cord injury rehabilitation research: patient priorities, current deficiencies and potential directions.* Disabil Rehabil, 2010. **32**(14): p. 1209-18.

10. Maynard, F.M., R.S. Karunas, and W.P. Waring, 3rd, *Epidemiology of spasticity following traumatic spinal cord injury.* Arch Phys Med Rehabil, 1990. **71**(8): p. 566-9.

11. Sekhon, L.H. and M.G. Fehlings, *Epidemiology, demographics, and pathophysiology of acute spinal cord injury.* Spine (Phila Pa 1976), 2001. **26**(24 Suppl): p. S2-12.

12. Eldahan, K.C. and A.G. Rabchevsky, *Autonomic dysreflexia after spinal cord injury: Systemic pathophysiology and methods of management.* Auton Neurosci, 2017.

13. Carlson, M., et al., *Lifestyle intervention for adults with spinal cord injury: Results of the USC-RLANRC Pressure Ulcer Prevention Study.* J Spinal Cord Med, 2017: p. 1-18.

14. Chen, Y., et al., *Obesity intervention in persons with spinal cord injury.* Spinal Cord, 2006. **44**(2): p. 82-91.

15. Farkas, G.J. and D.R. Gater, *Neurogenic obesity and systemic inflammation following spinal cord injury: a review.* J Spinal Cord Med, 2017: p. 1-10.

16. Saulino, M., *Spinal cord injury pain.* Phys Med Rehabil Clin N Am, 2014. **25**(2): p. 397-410.

17. Felix, E.R., Y. Cruz-Almeida, and E.G. Widerström-Noga, *Chronic pain after spinal cord injury: What characteristics make some pains more disturbing than others?* The Journal of Rehabilitation Research and Development, 2007. **44**(5): p. 703.

18. Sweis, R. and J. Biller, *Systemic Complications of Spinal Cord Injury.* Curr Neurol Neurosci Rep, 2017. **17**(2): p. 8.

19. Sun, X., et al., *Multiple organ dysfunction and systemic inflammation after spinal cord injury: a complex relationship.* J Neuroinflammation, 2016. **13**(1): p. 260.

20. Devivo, M.J., *Epidemiology of traumatic spinal cord injury: trends and future implications.* Spinal Cord, 2012. **50**(5): p. 365-72.

21. Chaovipoch, P., et al., *17beta-estradiol is protective in spinal cord injury in post- and pre-menopausal rats.* J Neurotrauma, 2006. **23**(6): p. 830-52.

22. Farooque, M., et al., *Gender-related differences in recovery of locomotor function after spinal cord injury in mice.* Spinal Cord, 2006. **44**(3): p. 182-7.

23. Datto, J.P., et al., *Female Rats Demonstrate Improved Locomotor Recovery and Greater Preservation of White and Gray Matter after Traumatic Spinal Cord Injury Compared to Males.* J Neurotrauma, 2015. **32**(15): p. 1146-57.

24. Datto, J.P., et al., *Does being female provide a neuroprotective advantage following spinal cord injury?* Neural Regen Res, 2015. **10**(10): p. 1533-6.

25. Cox, A., et al., *Nanoparticle Estrogen in Rat Spinal Cord Injury Elicits Rapid Anti-Inflammatory Effects in Plasma, Cerebrospinal Fluid, and Tissue.* J Neurotrauma, 2015. **32**(18): p. 1413-21.

26. Kachadroka, S., et al., *Effect of endogenous androgens on 17beta-estradiol-mediated protection after spinal cord injury in male rats.* J Neurotrauma, 2010. **27**(3): p. 611-26.

27. Olsen, M.L., et al., *Spinal cord injury causes a wide-spread, persistent loss of Kir4.1 and glutamate transporter 1: benefit of 17 beta-oestradiol treatment.* Brain, 2010. **133**(Pt 4): p. 1013-25.

28. Samantaray, S., et al., *Low dose estrogen prevents neuronal degeneration and microglial reactivity in an acute model of spinal cord injury: effect of dosing, route of administration, and therapy delay.* Neurochem Res, 2011. **36**(10): p. 1809-16.

29. Siriphorn, A., S. Chompoopong, and C.L. Floyd, *17beta-estradiol protects Schwann cells against H2O2-induced cytotoxicity and increases transplanted Schwann cell survival in a cervical hemicontusion spinal cord injury model.* J Neurochem, 2010. **115**(4): p. 864-72.

30. Siriphorn, A., et al., *Postinjury administration of 17beta-estradiol induces protection in the gray and white matter with associated functional recovery after cervical spinal cord injury in male rats.* J Comp Neurol, 2012. **520**(12): p. 2630-46.

31. Sribnick, E.A., et al., *Estrogen attenuated markers of inflammation and decreased lesion volume in acute spinal cord injury in rats.* J Neurosci Res, 2005. **82**(2): p. 283-93.

32. Yune, T.Y., et al., *Systemic administration of 17beta-estradiol reduces apoptotic cell death and improves functional recovery following traumatic spinal cord injury in rats.* J Neurotrauma, 2004. **21**(3): p. 293-306.

33. Breivik, H., et al., *Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment.* Eur J Pain, 2006. **10**(4): p. 287-333.

34. Gureje, O., et al., *Persistent pain and well-being: a World Health Organization Study in Primary Care.* Jama, 1998. **280**(2): p. 147-51.

35. Siddall, P.J., *Management of neuropathic pain following spinal cord injury: now and in the future.* Spinal Cord, 2009. **47**(5): p. 352-9.

36. Costigan, M., J. Scholz, and C.J. Woolf, *Neuropathic pain: a maladaptive response of the nervous system to damage.* Annu Rev Neurosci, 2009. **32**: p. 1-32.

37. Hulsebosch, C.E., et al., *Mechanisms of chronic central neuropathic pain after spinal cord injury.* Brain Res Rev, 2009. **60**(1): p. 202-13.

38. Lee, S., et al., *Central Neuropathic Pain in Spinal Cord Injury.* Critical reviews in physical and rehabilitation medicine, 2013. **25**(3-4): p. 159-172.

39. Zimmermann, M., *Pathobiology of neuropathic pain.* European Journal of Pharmacology, 2001. **429**: p. 5.

40. Latremoliere, A. and C.J. Woolf, *Central sensitization: a generator of pain hypersensitivity by central neural plasticity.* J Pain, 2009. **10**(9): p. 895-926.

41. Bouhassira, D., et al., *Prevalence of chronic pain with neuropathic characteristics in the general population.* Pain, 2008. **136**(3): p. 380-7.

42. Angelika EM Mautes, M.R.W., Frances Donovan, Linda J Noble, *Vascular Events After Spinal Cord Injury: Contribution to Secondary Pathogenesis.* Phys Ther, 2000. **80**: p. 17.

43. Hausmann, O.N., *Post-traumatic inflammation following spinal cord injury.* Spinal Cord, 2003. **41**(7): p. 369-78.

44. Oyinbo, C.A., *Secondary injury mechanisms in traumatic spinal cord injury:a nugget of this multiply cascade.* Acta Neurobiologiae Experimentalis, 2011. **71**: p. 19.

45. Charles H. tator, M.G.F., *Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms.* J Neurosurg, 1991. **75**: p. 12.

46. Eugene Park Alexander A. Velumian, a.M.G.F., *The Role of Excitotoxicity in Secondary Mechanisms of Spinal Cord Injury: A Review with an Emphasis on the Implications for White Matter Degeneration.* Journal of Neurotrauma, 2004. **21**(6): p. 21.

47. Zweier, J.L. and M.A.H. Talukder, *The role of oxidants and free radicals in reperfusion injury.* Cardiovascular Research, 2006. **70**(2): p. 181-190.

48. Hall, E.D., *Antioxidant Therapies for Acute Spinal Cord Injury.* Neurotherapeutics, 2011. **8**(2): p. 152-167.

49. Lobo, V., et al., *Free radicals, antioxidants and functional foods: Impact on human health.* Pharmacognosy Reviews, 2010. **4**(8): p. 118-126.

50. Liu, D., W. Thangnipon, and D.J. McAdoo, *Excitatory amino acids rise to toxic levels upon impact injury to the rat spinal cord.* Brain Res, 1991. **547**(2): p. 344-8.

51. Liu, D., et al., *Neurotoxicity of glutamate at the concentration released upon spinal cord injury.* Neuroscience, 1999. **93**(4): p. 1383-9.

52. Stout, A.K., et al., *Glutamate-induced neuron death requires mitochondrial calcium uptake.* Nat Neurosci, 1998. **1**(5): p. 366-73.

53. Pruss, H., et al., *Non-resolving aspects of acute inflammation after spinal cord injury (SCI): indices and resolution plateau.* Brain Pathol, 2011. **21**(6): p. 652-60.

54. Couillard-Despres, S., L. Bieler, and M. Vogl, *Pathophysiology of Traumatic Spinal Cord Injury*, in *Neurological Aspects of Spinal Cord Injury*. 2017, Springer. p. 503-528.

55. Jeffery, N.D. and W.F. Blakemore, *Locomotor deficits induced by experimental spinal cord demyelination are abolished by spontaneous remyelination.* Brain, 1997. **120**(1): p. 27-37.

56. Gledhill, R.F., B.M. Harrison, and W.I. McDonald, *Demyelination and remyelination after acute spinal cord compression.* Experimental Neurology, 1973. **38**(3): p. 472-487.

57. Fitch, M.T. and J. Silver, *CNS Injury, Glial Scars, and Inflammation: Inhibitory extracellular matrices and regeneration failure.* Experimental neurology, 2008. **209**(2): p. 294-301.

58. Fawcett, J.W. and R.A. Asher, *The glial scar and central nervous system repair.* Brain Res Bull, 1999. **49**(6): p. 377-91.

59. Esposito, E. and S. Cuzzocrea, *Anti-TNF therapy in the injured spinal cord.* Trends Pharmacol Sci, 2011. **32**(2): p. 107-15.

60. Xu, J., et al., *Methylprednisolone inhibition of TNF-alpha expression and NF-kB activation after spinal cord injury in rats.* Brain Res Mol Brain Res, 1998. **59**(2): p. 135-42.

61. Bennett, A.D., A.W. Everhart, and C.E. Hulsebosch, *Intrathecal administration of an NMDA or a non-NMDA receptor antagonist reduces mechanical but not thermal allodynia in a rodent model of chronic central pain after spinal cord injury.* Brain Res, 2000. **859**(1): p. 72-82.

62. Christoph, T., et al., *Silencing of vanilloid receptor TRPV1 by RNAi reduces neuropathic and visceral pain in vivo.* Biochem Biophys Res Commun, 2006. **350**(1): p. 238-43.

63. Chu, L.W., et al., *Atorvastatin prevents neuroinflammation in chronic constriction injury rats through nuclear NFkappaB downregulation in the dorsal root ganglion and spinal cord.* ACS Chem Neurosci, 2015. **6**(6): p. 889-98.

64. Costa, B., et al., *AM404, an inhibitor of anandamide uptake, prevents pain behaviour and modulates cytokine and apoptotic pathways in a rat model of neuropathic pain.* Br J Pharmacol, 2006. **148**(7): p. 1022-32.

65. Crown, E.D., et al., *Calcium/calmodulin dependent kinase II contributes to persistent central neuropathic pain following spinal cord injury.* Pain, 2012. **153**(3): p. 710-21.

66. Ellis, A., et al., *Systemic administration of propentofylline, ibudilast, and (+)-naltrexone each reverses mechanical allodynia in a novel rat model of central neuropathic pain.* J Pain, 2014. **15**(4): p. 407-21.

67. Griggs, R.B., et al., *Pioglitazone rapidly reduces neuropathic pain through astrocyte and nongenomic PPARgamma mechanisms.* Pain, 2015. **156**(3): p. 469-82.

68. Seltzer, Z., et al., *Modulation of neuropathic pain behavior in rats by spinal disinhibition and NMDA receptor blockade of injury discharge.* Pain, 1991. **45**(1): p. 69-75.

69. Zakir, H.M., et al., *Expression of TRPV1 channels after nerve injury provides an essential delivery tool for neuropathic pain attenuation.* PLoS One, 2012. **7**(9): p. e44023.

70. Zhou, C., et al., *Montelukast attenuates neuropathic pain through inhibiting p38 mitogen-activated protein kinase and nuclear factor-kappa B in a rat model of chronic constriction injury.* Anesth Analg, 2014. **118**(5): p. 1090-6.

71. Frankfurt, M., E. Fuchs, and W. Wuttke, *Sex differences in gamma-aminobutyric acid and glutamate concentrations in discrete rat brain nuclei.* Neurosci Lett, 1984. **50**(1-3): p. 245-50.

72. Sorge, R.E., et al., *Different immune cells mediate mechanical pain hypersensitivity in male and female mice.* Nat Neurosci, 2015. **18**(8): p. 1081-3.

73. Fehlings, M.G. and R.G. Perrin, *The timing of surgical intervention in the treatment of spinal cord injury: a systematic review of recent clinical evidence.* Spine (Phila Pa 1976), 2006. **31**(11 Suppl): p. S28-35; discussion S36.

74. Burney, R.E., et al., *Incidence, characteristics, and outcome of spinal cord injury at trauma centers in North America.* Arch Surg, 1993. **128**(5): p. 596-9.

75. Young, W., *Spinal cord contusion models.* Prog Brain Res, 2002. **137**: p. 231-55.

76. Cheriyan, T., et al., *Spinal cord injury models: a review.* Spinal Cord, 2014. **52**(8): p. 588-95.

77. Sharif-Alhoseini, M., et al., *Animal models of spinal cord injury: a systematic review.* Spinal Cord, 2017. **55**(8): p. 714-721.

78. Rosenzweig, E.S. and J.W. McDonald, *Rodent models for treatment of spinal cord injury: research trends and progress toward useful repair.* Curr Opin Neurol, 2004. **17**(2): p. 121-31.

79. Kjell, J. and L. Olson, *Rat models of spinal cord injury: from pathology to potential therapies.* Disease Models & Mechanisms, 2016. **9**(10): p. 1125-1137.

80. Wen, J., et al., *A Consistent, Quantifiable, and Graded Rat Lumbosacral Spinal Cord Injury Model.* Journal of Neurotrauma, 2015. **32**(12): p. 875-892.

81. Eaton, M., *Common animal models for spasticity and pain.* J Rehabil Res Dev, 2003. **40**(4 Suppl 1): p. 41-54.

82. Dunham, K.A., et al., *Characterization of a graded cervical hemicontusion spinal cord injury model in adult male rats.* J Neurotrauma, 2010. **27**(11): p. 2091-106.

83. Schneider, L.E., et al., *Application of the Rat Grimace Scale as a Marker of Supraspinal Pain Sensation after Cervical Spinal Cord Injury.* J Neurotrauma, 2017.

84. Thompson, F.J. and P. Bose, *Chapter L4 - Rat Spinal Cord Contusion Model of Spasticity*, in *Animal Models of Movement Disorders*, M. LeDoux, Editor. 2005, Academic Press: Burlington. p. 699-712.

85. Khan, T., et al., *Animal models of spinal cord contusion injuries.* Lab Anim Sci, 1999. **49**(2): p. 161-72.

86. Cullinan, J.P.H.a.W.E., *Neurocircuitry of stress: central control of the hypothalamo–pituitary–adrenocortical axis.* Trends Neurosci, 1997. **20**: p. 7.

87. McEwen, B.S., *Physiology and neurobiology of stress and adaptation: central role of the brain.* Physiol Rev, 2007. **87**(3): p. 873-904.

88. Herman, J.P., *Neural control of chronic stress adaptation.* Front Behav Neurosci, 2013. **7**: p. 61.

89. Sorrells, S.F. and R.M. Sapolsky, *An inflammatory review of glucocorticoid actions in the CNS.* Brain Behav Immun, 2007. **21**(3): p. 259-72.

90. Galigniana, N.M., et al., *Regulation of the glucocorticoid response to stress-related disorders by the Hsp90-binding immunophilin FKBP51.* J Neurochem, 2012. **122**(1): p. 4-18.

91. Hoeijmakers, L., et al., *Depletion of FKBP51 in female mice shapes HPA axis activity.* PLoS One, 2014. **9**(4): p. e95796.

92. Jaaskelainen, T., H. Makkonen, and J.J. Palvimo, *Steroid up-regulation of FKBP51 and its role in hormone signaling.* Curr Opin Pharmacol, 2011. **11**(4): p. 326-31.

93. Wochnik, G.M., et al., *FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells.* J Biol Chem, 2005. **280**(6): p. 4609-16.

94. Babb, J.A., Masini, C. V., Day, H. E. and Campeau, S., *Sex differences in activated corticotropin-releasing factor neurons within stress-related neurocircuitry and hypothalamic-pituitary-adrenocortical axis hormones following restraint in rats.* Neuroscience, 2013. **234**: p. 40-52.

95. Glenn T. Livezey, J.M.M.a.W.H.V., *PLASMA NOREPINEPHRINE, EPINEPHRINE AND CORTICOSTERONE STRESS RESPONSES TO RESTRAINT IN INDIVIDUAL MALE AND FEMALE RATS, AND THEIR CORRELATIONS.* Neurosci Lett, 1985. **62**: p. 6.

96. G. Jean Kant, R.H.L., Bradford N. Bunnell, Edward H. Mougey, Lee l. Pennington and James l. Meyerhoff, *COMPARISON OF STRESS RESPONSE IN MALE AND FEMALE RATS: PITUITARY CYCLIC AMP AND PLASMA PROLACTIN, GROWTH HORMONE AND CORTICOSTERONE.* PSYCHONEUROENDOCRINO, 1983. **8**: p. 8.

97. Goel, N., et al., *Sex differences in the HPA axis.* Compr Physiol, 2014. **4**(3): p. 1121-55.

98. Anisman, H. and K. Matheson, *Stress, depression, and anhedonia: caveats concerning animal models.* Neurosci Biobehav Rev, 2005. **29**(4-5): p. 525-46.

99. Abbott, B.B., L.S. Schoen, and P. Badia, *Predictable and unpredictable shock: behavioral measures of aversion and physiological measures of stress.* Psychol Bull, 1984. **96**(1): p. 45-71.

100. Campbell, T., et al., *Coping strategies in male and female rats exposed to multiple stressors.* Physiol Behav, 2003. **78**(3): p. 495-504.

101. Checkley, S., *The neuroendocrinology of depression and chronic stress.* Br Med Bull, 1996. **52**(3): p. 597-617.

102. Bali, A. and A.S. Jaggi, *Electric foot shock stress: a useful tool in neuropsychiatric studies.* Rev Neurosci, 2015. **26**(6): p. 655-77.

103. Bali, A. and A.S. Jaggi, *Electric foot shock stress adaptation: Does it exist or not?* Life Sci, 2015. **130**: p. 97-102.

104. Black, L.V., T.J. Ness, and M.T. Robbins, *Effects of oxytocin and prolactin on stress-induced bladder hypersensitivity in female rats.* J Pain, 2009. **10**(10): p. 1065-72.

105. Kumar, V., Z.A. Bhat, and D. Kumar, *Animal models of anxiety: a comprehensive review.* J Pharmacol Toxicol Methods, 2013. **68**(2): p. 175-83.

106. Robbins, M.T. and T.J. Ness, *Footshock-induced urinary bladder hypersensitivity: role of spinal corticotropin-releasing factor receptors.* J Pain, 2008. **9**(11): p. 991-8.

107. Toth, I. and I.D. Neumann, *Animal models of social avoidance and social fear.* Cell Tissue Res, 2013. **354**(1): p. 107-18.

108. Robbins, M.T., et al., *Footshock stress differentially affects responses of two subpopulations of spinal dorsal horn neurons to urinary bladder distension in rats.* Brain Res, 2011. **1386**: p. 118-26.

109. Anisman, H., et al., *Psychogenic, neurogenic, and systemic stressor effects on plasma corticosterone and behavior: mouse strain-dependent outcomes.* Behav Neurosci, 2001. **115**(2): p. 443-54.

110. Mestre, H., et al., *Lewis, Fischer 344, and sprague-dawley rats display differences in lipid peroxidation, motor recovery, and rubrospinal tract preservation after spinal cord injury.* Front Neurol, 2015. **6**: p. 108.

111. Schmitt, C., et al., *Changes in spinal cord injury-induced gene expression in rat are strain-dependent.* Spine J, 2006. **6**(2): p. 113-9.

112. Firdaus S. Dhabhar, B.S.M., Robert L. Spencer, *Stress response, adrenal steroid receptor levels and corticosteroid-binding globulin levels - a comparison between Sprague Dawley, Fischer 344 and Lewis rats.* Brain Research, 1993. **616**: p. 10.

113. Etingen, B., et al., *Examining the relationship between post-traumatic stress disorder and social participation among Veterans with spinal cord injuries and disorders.* Disabil Rehabil, 2017: p. 1-7.

114. Kip, K.E., et al., *Accelerated Resolution Therapy for treatment of pain secondary to symptoms of combat-related posttraumatic stress disorder.* Eur J Psychotraumatol, 2014. **5**.

115. A.J. Hampson, M.G., J. Axelrod, and D. Wink, *Cannabidiol and (-)delta9-tetrahydrocannabinol are neuroprotective antioxidants.* Proc Natl Acad Sci U S A, 1998. **95**: p. 6.

116. A. W. Zuardi, I.S., E. Finkelfarb, and I. G. Karniol, *Action of cannabidiol on the anxiety and other effects produced by delta THC in normal subjects.* Psychopharmacology, 1982. **76**: p. 6.

117. Agurell, S., et al., *Pharmacokinetics and metabolism of delta 1-tetrahydrocannabinol and other cannabinoids with emphasis on man.* Pharmacol Rev, 1986. **38**(1): p. 21-43.

118. Bergamaschi, M.M., et al., *Safety and side effects of cannabidiol, a Cannabis sativa constituent.* Curr Drug Saf, 2011. **6**(4): p. 237-49.

119. Costa, B., et al., *Vanilloid TRPV1 receptor mediates the antihyperalgesic effect of the nonpsychoactive cannabinoid, cannabidiol, in a rat model of acute inflammation.* Br J Pharmacol, 2004. **143**(2): p. 247-50.

120. Costa, B., et al., *The non-psychoactive cannabis constituent cannabidiol is an orally effective therapeutic agent in rat chronic inflammatory and neuropathic pain.* Eur J Pharmacol, 2007. **556**(1-3): p. 75-83.

121. Hampson, A.J., et al., *Cannabidiol and (-)Delta9-tetrahydrocannabinol are neuroprotective antioxidants.* Proc Natl Acad Sci U S A, 1998. **95**(14): p. 8268-73.

122. Johnson, J.R., et al., *Multicenter, double-blind, randomized, placebo-controlled, parallel-group study of the efficacy, safety, and tolerability of THC:CBD extract and THC extract in patients with intractable cancer-related pain.* J Pain Symptom Manage, 2010. **39**(2): p. 167-79.

123. *Cannabis-based medicines--GW pharmaceuticals: high CBD, high THC, medicinal cannabis--GW pharmaceuticals, THC:CBD.* Drugs R D, 2003. **4**(5): p. 306-9.

124. Smith, P.M. and N.D. Jeffery, *Histological and ultrastructural analysis of white matter damage after naturally-occurring spinal cord injury.* Brain Pathol, 2006. **16**(2): p. 99-109.

125. Hagen, E.M. and T. Rekand, *Management of Neuropathic Pain Associated with Spinal Cord Injury.* Pain and Therapy, 2015. **4**(1): p. 51-65.

126. Werhagen, L., et al., *Neuropathic pain after traumatic spinal cord injury--relations to gender, spinal level, completeness, and age at the time of injury.* Spinal Cord, 2004. **42**(12): p. 665-73.

127. Siddall, P.J., et al., *A longitudinal study of the prevalence and characteristics of pain in the first 5 years following spinal cord injury.* Pain, 2003. **103**(3): p. 249-257.

128. Hagenbach, U., et al., *The treatment of spasticity with Delta9-tetrahydrocannabinol in persons with spinal cord injury.* Spinal Cord, 2007. **45**(8): p. 551-62.

129. Harvey, P.J., M. Gorassini, and D.J. Bennett, *Chapter L3 - The Spastic Rat with Sacral Spinal Cord Injury*, in *Animal Models of Movement Disorders*, M. LeDoux, Editor. 2005, Academic Press: Burlington. p. 691-697.

130. Skold, C., R. Levi, and A. Seiger, *Spasticity after traumatic spinal cord injury: nature, severity, and location.* Arch Phys Med Rehabil, 1999. **80**(12): p. 1548-57.

131. van Gorp, S., et al., *Translation of the rat thoracic contusion model; part 1-supraspinally versus spinally mediated pain-like responses and spasticity.* Spinal Cord, 2014. **52**(7): p. 524-8.

132. Matsumiya, L.C., et al., *Using the Mouse Grimace Scale to reevaluate the efficacy of postoperative analgesics in laboratory mice.* J Am Assoc Lab Anim Sci, 2012. **51**(1): p. 42-9.

133. Sotocinal, S.G., et al., *The Rat Grimace Scale: a partially automated method for quantifying pain in the laboratory rat via facial expressions.* Mol Pain, 2011. **7**: p. 55.

134. Langford, D.J., et al., *Coding of facial expressions of pain in the laboratory mouse.* Nat Methods, 2010. **7**(6): p. 447-9.

135. Tatman, A., et al., *Development of a modified paediatric coma scale in intensive care clinical practice.* Arch Dis Child, 1997. **77**(6): p. 519-21.

136. Trotman, C.A. and J.J. Faraway, *Sensitivity of a method for the analysis of facial mobility. II. Interlandmark separation.* Cleft Palate Craniofac J, 1998. **35**(2): p. 142-53.

137. Williams, A.L., et al., *The behavioral pain response to heelstick in preterm neonates studied longitudinally: description, development, determinants, and components.* Early Hum Dev, 2009. **85**(6): p. 369-74.

138. Putatunda, R., et al., *Chronic at-level thermal hyperalgesia following rat cervical contusion spinal cord injury is accompanied by neuronal and astrocyte activation and loss of the astrocyte glutamate transporter, GLT1, in superficial dorsal horn.* Brain Res, 2014. **1581**: p. 64-79.

139. Coderre, T.J., R.W. Grimes, and R. Melzack, *Deafferentation and chronic pain in animals: an evaluation of evidence suggesting autotomy is related to pain.* Pain, 1986. **26**(1): p. 61-84.

140. Kauppila, T., *Correlation between autotomy-behavior and current theories of neuropathic pain.* Neuroscience & Biobehavioral Reviews, 1998. **23**: p. 10.

141. Xu, X.J., et al., *Chronic pain-related syndrome in rats after ischemic spinal cord lesion: a possible animal model for pain in patients with spinal cord injury.* Pain, 1992. **48**(2): p. 279-90.

142. Bardin, L., et al., *Chronic restraint stress induces mechanical and cold allodynia, and enhances inflammatory pain in rat: Relevance to human stress-associated painful pathologies.* Behav Brain Res, 2009. **205**(2): p. 360-6.

143. Bravo, L., et al., *Social stress exacerbates the aversion to painful experiences in rats exposed to chronic pain: the role of the locus coeruleus.* Pain, 2013. **154**(10): p. 2014-23.

144. Yamamoto, K., et al., *Transient receptor potential ankyrin 1 that is induced in dorsal root ganglion neurons contributes to acute cold hypersensitivity after oxaliplatin administration.* Mol Pain, 2015. **11**: p. 69.

145. Zhao, M., et al., *Acute cold hypersensitivity characteristically induced by oxaliplatin is caused by the enhanced responsiveness of TRPA1 in mice.* Mol Pain, 2012. **8**: p. 55.

146. Gees, M., B. Colsoul, and B. Nilius, *The role of transient receptor potential cation channels in Ca2+ signaling.* Cold Spring Harb Perspect Biol, 2010. **2**(10): p. a003962.

147. Pedersen, S.F., G. Owsianik, and B. Nilius, *TRP channels: an overview.* Cell Calcium, 2005. **38**(3-4): p. 233-52.

148. Kim, Y.S., et al., *Expression of transient receptor potential ankyrin 1 (TRPA1) in the rat trigeminal sensory afferents and spinal dorsal horn.* J Comp Neurol, 2010. **518**(5): p. 687-98.

149. Patapoutian, A., S. Tate, and C.J. Woolf, *Transient receptor potential channels: targeting pain at the source.* Nature reviews. Drug discovery, 2009. **8**(1): p. 55-68.

150. Binder, A., et al., *Transient receptor potential channel polymorphisms are associated with the somatosensory function in neuropathic pain patients.* PLoS One, 2011. **6**(3): p. e17387.

151. Ryan, D., et al., *Cannabidiol targets mitochondria to regulate intracellular Ca2+ levels.* J Neurosci, 2009. **29**(7): p. 2053-63.

152. Giudice, E.D., et al., *Cannabidiol, unlike synthetic cannabinoids, triggers activation of RBL-2H3 mast cells.* J Leukoc Biol, 2007. **81**(6): p. 1512-22.

153. Aloisi, A.M., *Gonadal hormones and sex differences in pain reactivity.* Clin J Pain, 2003. **19**(3): p. 168-74.

154. Aloisi, A.M. and M. Bonifazi, *Sex hormones, central nervous system and pain.* Horm Behav, 2006. **50**(1): p. 1-7.

155. Wiesenfeld-Hallin, Z., *Sex differences in pain perception.* Gend Med, 2005. **2**(3): p. 137-45.

156. Edwards, R.R., et al., *Pain tolerance as a predictor of outcome following multidisciplinary treatment for chronic pain: differential effects as a function of sex.* Pain, 2003. **106**(3): p. 419-26.

157. Kuba, T. and V. Quinones-Jenab, *The role of female gonadal hormones in behavioral sex differences in persistent and chronic pain: clinical versus preclinical studies.* Brain Res Bull, 2005. **66**(3): p. 179-88.

158. Gatson, J.W., et al., *Estrogen treatment following severe burn injury reduces brain inflammation and apoptotic signaling.* Journal of Neuroinflammation, 2009. **6**: p. 30-30.

159. Samantaray, S., et al., *Administration of low dose estrogen attenuates gliosis and protects neurons in acute spinal cord injury in rats.* J Neurochem, 2016. **136**(5): p. 1064-73.

160. Deiana, S., et al., *Plasma and brain pharmacokinetic profile of cannabidiol (CBD), cannabidivarine (CBDV), Delta(9)-tetrahydrocannabivarin (THCV) and cannabigerol (CBG) in rats and mice following oral and intraperitoneal administration and CBD action on obsessive-compulsive behaviour.* Psychopharmacology (Berl), 2012. **219**(3): p. 859-73.

161. Grotenhermen, F., *Pharmacokinetics and Pharmacodynamics of Cannabinoids.* Clin Pharmacokinet, 2003. **42**(4): p. 34.

162. Hill, T.D., et al., *Cannabidivarin-rich cannabis extracts are anticonvulsant in mouse and rat via a CB1 receptor-independent mechanism.* Br J Pharmacol, 2013. **170**(3): p. 679-92.

163. Blackburn-Munro, G., *Hypothalamo-pituitary-adrenal axis dysfunction as a contributory factor to chronic pain and depression.* Curr Pain Headache Rep, 2004. **8**(2): p. 116-24.

164. Blackburn-Munro, G. and R.E. Blackburn-Munro, *Chronic pain, chronic stress and depression: coincidence or consequence?* J Neuroendocrinol, 2001. **13**(12): p. 1009-23.

165. Ulrich-Lai, Y.M., et al., *Limbic and HPA axis function in an animal model of chronic neuropathic pain.* Physiol Behav, 2006. **88**(1-2): p. 67-76.

166. Griep, E.N., et al., *Function of the hypothalamic-pituitary-adrenal axis in patients with fibromyalgia and low back pain.* J Rheumatol, 1998. **25**(7): p. 1374-81.

167. Gaab, J., et al., *Reduced reactivity and enhanced negative feedback sensitivity of the hypothalamus-pituitary-adrenal axis in chronic whiplash-associated disorder.* Pain, 2005. **119**(1-3): p. 219-24.

168. Galli, U., et al., *Enhanced negative feedback sensitivity of the hypothalamus-pituitary-adrenal axis in chronic myogenous facial pain.* Eur J Pain, 2009. **13**(6): p. 600-5.

169. McBeth, J., et al., *Hypothalamic-pituitary-adrenal stress axis function and the relationship with chronic widespread pain and its antecedents.* Arthritis Res Ther, 2005. **7**(5): p. R992-r1000.

170. Generaal, E., et al., *Reduced hypothalamic-pituitary-adrenal axis activity in chronic multi-site musculoskeletal pain: partly masked by depressive and anxiety disorders.* BMC Musculoskeletal Disorders, 2014. **15**: p. 227-227.

171. Gomez, F., et al., *Hypothalamic-pituitary-adrenal response to chronic stress in five inbred rat strains: Differential responses are mainly located at the adrenocortical level.* Neuroendocrinology, 1996. **63**(4): p. 327-337.

172. Vasquez, C.E., et al., *NMDA receptor dysregulation in chronic state: a possible mechanism underlying depression with BDNF downregulation.* Neurochem Int, 2014. **79**: p. 88-97.

173. Alexander, J.K., et al., *Stress exacerbates neuropathic pain via glucocorticoid and NMDA receptor activation.* Brain Behav Immun, 2009. **23**(6): p. 851-60.

174. *Frontiers in Neuroscience*, in *Biology of the NMDA Receptor*, A.M. Van Dongen, Editor. 2009, CRC Press/Taylor & Francis Taylor & Francis Group, LLC.: Boca Raton (FL).

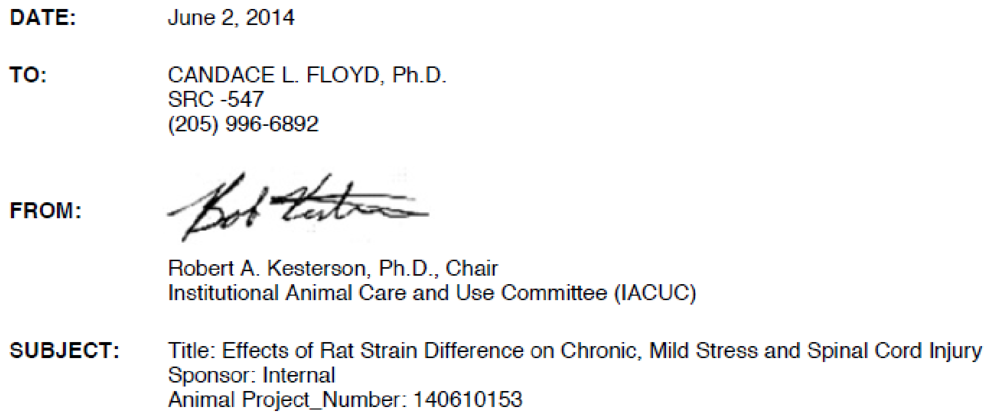
APPENDIX A

IACUC APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL



DATE: June 2, 2014

TO: CANDACE L. FLOYD, Ph.D.
SRC -547
(205) 996-6892

FROM:

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Effects of Rat Strain Difference on Chronic, Mild Stress and Spinal Cord Injury
Sponsor: Internal
Animal Project_Number: 140610153

As of June 2, 2014 the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and number of animals:

Species	Use Category	Number In Category
Rats	B	40
Rats	C	40

Animal use must be renewed by June 1, 2015. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 140610153 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee (IACUC)
CH19 Suite 403
933 19th Street South
(205) 934-7692
FAX (205) 934-1188

Mailing Address:
CH19 Suite 403
1530 3rd Ave S
Birmingham, AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

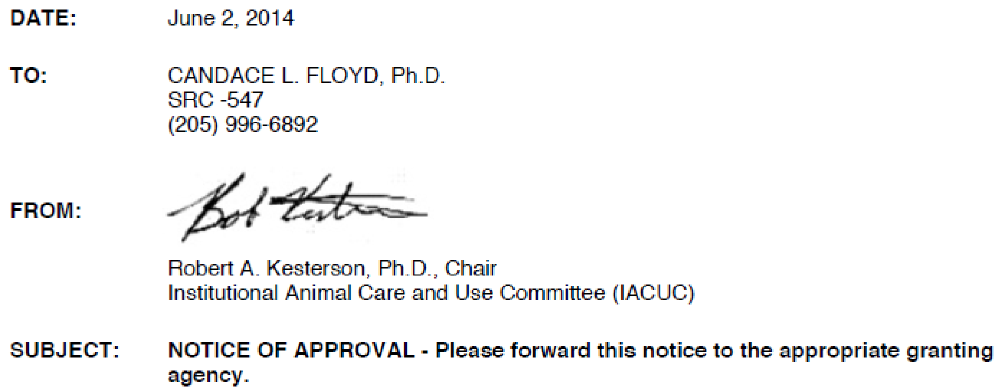
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: June 2, 2014

TO: CANDACE L. FLOYD, Ph.D.
SRC -547
(205) 996-6892

FROM:



Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

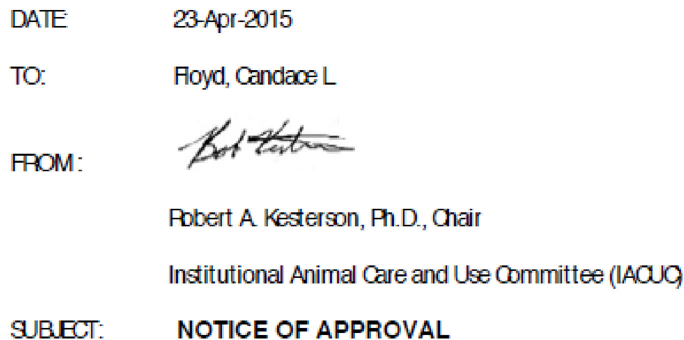
The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on June 2, 2014.

Title: Effects of Rat Strain Difference on Chronic, Mild Stress and Spinal Cord Injury
Sponsor: Internal

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC)
CH19 Suite 403
933 19th Street South
(205) 934-7692
FAX (205) 934-1188

Mailing Address:
CH19 Suite 403
1530 3rd Ave S
Birmingham, AL 35294-0019



MEMORANDUM

DATE: 23-Apr-2015

TO: Floyd, Candace L

FROM:

Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 23-Apr-2015.

Protocol PI: Floyd, Candace L

Title: Effects of Rat Strain Difference on Chronic, Mild Stress and Spinal Cord Injury

Sponsor: UAB DEPARTMENT

Animal Project Number (APN): IACUC-10153

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
CH19 Suite 403 | CH19 Suite 403
933 19th Street South | 1530 3rd Ave S
(205) 934-7692 | Birmingham, AL 35294-0019
FAX (205) 934-1188 |

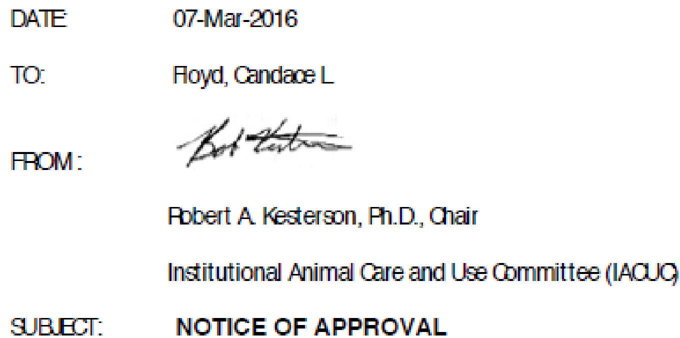
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 07-Mar-2016

TO: Floyd, Candace L

FROM:



Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 07-Mar-2016.

Protocol PI: Floyd, Candace L

Title: Evaluation of Cannabidiol to Treat Chronic Pain After Spinal Cord Injury

Sponsor: CONQUER PARALYSIS NOW

Animal Project Number (APN): IACUC-20299

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
CH19 Suite 403 | CH19 Suite 403
933 19th Street South | 1530 3rd Ave S
(205) 934-7692 | Birmingham, AL 35294-0019
FAX (205) 934-1188 |

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 11-Jan-2017

TO: Floyd, Candace L

FROM:

Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 11-Jan-2017.

Protocol PI: Floyd, Candace L

Title: Evaluation of Cannabidiol to Treat Chronic Pain After Spinal Cord Injury

Sponsor: CONQUER PARALYSIS NOW

Animal Project Number (APN): IACUC-20299

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
CH19 Suite 403 | CH19 Suite 403
933 19th Street South | 1530 3rd Ave S
(205) 934-7692 | Birmingham, AL 35294-0019
FAX (205) 934-1188 |