Targeting monoacylglycerol lipase for the reversal and prevention of paclitaxel-induced allodynia in mice

Zachary Curry
Virginia Commonwealth University

Follow this and additional works at: https://scholarscompass.vcu.edu/etd

Part of the Pharmacology Commons

© The Author

Downloaded from
https://scholarscompass.vcu.edu/etd/5274

This Dissertation is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.
Targeting monoacylglycerol lipase for the reversal and prevention of paclitaxel-induced allodynia in mice

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

By

Zachary Adam Curry

Bachelor of Science, Winthrop University, 2012

Director: Aron H. Lichtman, Ph.D.
Professor, Department of Pharmacology and Toxicology
Associate Dean of Research and Graduate Studies, School of Pharmacy
Virginia Commonwealth University

Virginia Commonwealth University
Richmond, Virginia
February 2018
Acknowledgements

Words cannot express my gratitude to everyone who has supported me through my Ph.D. training. In particular, I want to thank my mentor, Dr. Aron Lichtman, who encouraged me to join his laboratory even while I was new to neuroscience and pharmacology. In addition to supervising my research training and providing critical feedback on my projects, he took the time to mentor me in an independent study course on the endogenous cannabinoid system in pain. I am deeply indebted to him for his mentorship and advocacy on my behalf. I also want to thank Dr. Jenny Wilkerson, who trained me in mechanical allodynia and immunohistochemistry and who supported my growth as a scientist. Additionally, I am deeply grateful for the help and support of other current and former Lichtman lab members, including Dr. Giulia Donvito, Dr. Travis Grim, Dr. Allen Owens, Lesley Schurman, Mohammed Mustafa, Rehab Abdullah and Anthony Morales.

Several leaders from the M.D./Ph.D. program and the Department of Pharmacology and Toxicology at VCU, in particular Dr. Michael Donnenberg, Dr. Ross Mikkelsen, Dr. Gordon Archer and Dr. William Dewey, have provided unwavering support through each phase of my dual-degree training. I would also like to thank my committee members who have served as second mentors to me—in particular, Dr. Imad Damaj. In addition to the friendship he and his lab have shown me, they have contributed to my dissertation on a practical level. Dr. Damaj has
spent many hours with me as a second advisor and also included me in a course he was teaching on the fundamentals of pain physiology. Dr. Deniz Bagdas and Wisam Toma, both of the Damaj lab, trained me in mechanical allodynia and the conditioned place preference paradigm. Dr. Bagdas made a significant contribution to my project by conducting the conditioned place preference paradigm studies. Additionally, I would like to thank Dr. Steven Negus, who allowed me to work in his laboratory at the beginning of my Ph.D. program. Dr. John Bigbee has also been especially helpful, providing critical feedback and supporting my immunohistochemistry training. Dr. Guy Cabral has provided insightful feedback into the design of my studies and plans for additional experiments.

Lastly, I am indebted to my friends and family who have provided the social support needed to complete my Ph.D. training. In particular, I thank my wonderful wife of two years, Courtney Curry, who has tirelessly supported me through each season of my Ph.D. training. I am richly blessed to have found my better half. I also want to thank each and every member of my family, including my parents and grandparents, who have provided countless hours of advice and support. Lastly, I thank Dr. William Marks for his friendship and wise advice.
# Table of Contents

List of Tables ............................................................................................................................ vii

List of Figures ............................................................................................................................. viii

List of Abbreviations .................................................................................................................. x

Abstract ....................................................................................................................................... xiii

Chapter 1: Introduction ................................................................................................................. 1

The Endogenous Cannabinoid System: Introduction ................................................................. 1

The Endogenous Cannabinoid System: Cannabinoid Receptors .............................................. 3

The Endogenous Cannabinoid System: Anandamide and 2-arachidonoylglycerol ................. 5

The Endogenous Cannabinoid System: Enzymatic Regulation of Anandamide ...................... 8

The Endogenous Cannabinoid System: Enzymatic Regulation of 2-arachidonoylglycerol ... 10

The Endogenous Cannabinoid System: Anti-Cancer Effects .................................................. 14

The Endogenous Cannabinoid System: Current Therapeutic Indications and Potential Analgesic Use ......................................................................................................................... 17

Chemotherapy-Induced Peripheral Neuropathies: A Clinical Problem ................................. 19

Paclitaxel, Cisplatin and Vincristine: Chemotherapeutic Mechanisms of Action................. 22

Animal Models of CIPN: Behavioral Changes to Mechanical, Thermal and Cold Stimuli .... 23

Changes in Affective Behaviors from CIPN ............................................................................... 25
Rationale for Focus on Paclitaxel CIPN ................................................................. 28
Paclitaxel CIPN: Peripheral Toxicity................................................................. 29
Paclitaxel CIPN: Dorsal Root Ganglia Toxicity............................................. 30
Paclitaxel CIPN: Spinal Dorsal Horn Toxicity............................................... 33
The Endogenous Cannabinoid System: Potential Targets to Treat CIPN........... 35
The Endogenous Cannabinoid System: Potential Targets to Prevent CIPN........ 41
Hypothesis ................................................................................................. 48
Mouse Model of Paclitaxel CIPN ................................................................. 48
Selection of MAGL Inhibitors ..................................................................... 49

Chapter 2 Overview: Monoacylglycerol lipase inhibitors reverse paclitaxel-induced nociceptive behavior and proinflammatory markers in a mouse model of chemotherapy-induced neuropathy ...................................................... 49

Chapter 3 Overview: Monoacylglycerol lipase inhibitors prevent the development of paclitaxel-induced mechanical allodynia in mice ................................................................. 51

Chapter 2: Monoacylglycerol lipase inhibitors reverse paclitaxel-induced nociceptive behavior and proinflammatory markers in a mouse model of chemotherapy-induced neuropathy ...................................................... 52

Chapter 3: Monoacylglycerol lipase inhibitors prevent the development of paclitaxel-induced mechanical allodynia in mice ................................................................. 98

Chapter 4: General Discussion ................................................................. 119

Monoacylglycerol lipase inhibitors reverse mechanical allodynia in a mouse model of paclitaxel CIPN ................................................................................................. 122

The anti-allodynic effects of monoacylglycerol lipase inhibitors are CB1- and CB2-dependent ................................................................................................. 124

Low-dose JZL184 produces anti-allodynic effects with repeated treatment and elevates spinal 2-AG levels ................................................................................................. 126

The impact of JZL184 treatment on anandamide, arachidonic acid and other spinal lipids ................................................................................................. 128
Limitations of mechanical allodynia as a measure of paclitaxel CIPN .......................... 130
MJN110 produces a conditioned place preference in paclitaxel-treated mice ............... 133
Monoacylglycerol lipase inhibitors reverse markers of paclitaxel-induced inflammation in the lumbar spinal cord and associated DRG ................................................................. 136
Monoacylglycerol lipase inhibitors do not affect the chemotherapeutic efficacy of paclitaxel in cell lines of non-small cell lung cancer .............................................................. 139
MAGL inhibition prevents the development of paclitaxel-induced mechanical allodynia... 140
Overall Conclusions ........................................................................................................ 144
Future Directions ............................................................................................................ 145
List of References .......................................................................................................... 152
Vita ................................................................................................................................. 189
List of Tables

Table 1: Review of literature demonstrating reversal of established allodynia or hyperalgesia in rodent models of CIPN by drugs targeting the endogenous cannabinoid system .................. 37

Table 2: Review of literature demonstrating prevention of allodynia and hyperalgesia behaviors in rodent models of CIPN by drugs targeting the endogenous cannabinoid system .... 45
List of Figures

Figure 1. Overview of the endogenous cannabinoid system.........................................................2
Figure 2. Markers of paclitaxel-induced allodynia by anatomic location......................................28
Figure 3. Diagram of hypothesis ....................................................................................................48
Figure 4. Paclitaxel induces a significant mechanical allodynia..................................................69
Figure 5. MAGL inhibitors significantly reverse mechanical allodynia in paclitaxel-treated mice ..........................................................................................................................70
Figure 6. MAGL inhibition does not enhance or depress paw withdrawal thresholds in control mice lacking paclitaxel ..................................................................................................71
Figure 7. ABHD6 inhibition does not alter paw withdrawal thresholds...........................................72
Figure 8. The anti-allodynic effects of MAGL inhibitors require CB₁ receptor activation...........74
Figure 9. The anti-allodynic effects of MAGL inhibitors require CB₂ receptor activation..........76
Figure 10. MJN110 attenuates paclitaxel-induced MCP-1 expression in the dorsal root ganglia .................................................................................................................................79
Figure 11. MJN110 attenuates paclitaxel-induced MCP-1 expression in the spinal dorsal horn .................................................................................................................................80
Figure 12. MJN110 attenuates paclitaxel-induced phospho-p38 MAPK expression in the dorsal root ganglia ......................................................................................................................81
Figure 13. Paclitaxel does not increase phospho-p38 expression in the spinal dorsal horn ..........82
Figure 14. Qualitative confocal microscopy of MCP-1 and phospho-p38 MAPK co-
localization in dorsal root ganglia cells ................................................................. 83

Figure 15. Paclitaxel (Pac)-treated mice used in the repeated administration study developed a significant mechanical allodynia compared to no paclitaxel vehicle control (Veh) mice after a cycle of paclitaxel ........................................................................... 85

Figure 16. Acute and repeated administration of 4 and 40 mg/kg JZL184 produce differential effects on paclitaxel-induced allodynia ................................................................. 86

Figure 17. Acute versus repeated administration of JZL184 (4 or 40 mg/kg) on 2-AG, anandamide (AEA), arachidonic acid, Prostaglandin D2 (PGD2) in spinal cord.. 87

Figure 18. MJN110 leads to the development of CPP in mice that received a cycle of paclitaxel, but not in mice that received a cycle of vehicle ................................................. 88

Figure 19. Paclitaxel-treated mice used in the CPP paradigm developed a significant mechanical allodynia compared to no paclitaxel vehicle control mice after paclitaxel treatment .................................................................................. 89

Figure 20. JZL184 does not stimulate non-small cell lung cancer (NSCLC) cell proliferation alone or interfere with paclitaxel (Pac, 50nM)-induced growth inhibition of A549 or H460 cells .......................................................................... 90

Figure 21. JZL184 does not affect paclitaxel-induced apoptosis of non-small cell lung cancer (NSCLC) cells ............................................................................................ 91

Figure 22. Experimental design for the prevention of paclitaxel-induced mechanical allodynia .................................................................................................................. 106

Figure 23. JZL184 prevents the development of, or enhances recovery from, paclitaxel-induced mechanical allodynia .................................................................................. 107

Figure 24. MJN110 prevents the development of paclitaxel-induced mechanical allodynia one week after paclitaxel treatment ................................................................. 108

Figure 25. Prevention of paclitaxel-induced mechanical allodynia is CB1 receptor-Independent ................................................................................................................. 110
List of Abbreviations

[^35S]GTPγS guanosine [35S]5'-O-[gamma-thio]triphasphate
2-AG 2-arachidonoylglycerol
ABHD alpha/beta-hydrolase domain
AEA anandamide
AM1241 1-(methylpiperidin-2-ylmethyl)-3-(2-iodo-5-nitrobenzoyl)indole
AM1710 3-(1,1-dimethylheptyl)-1-hydroxy-9-methoxy-6H-benzo[c]chromene-6-one
AM1714 1,9-Dihydroxy-3-(1,1-dimethylheptyl)-6H-benzo[c]chromene-6-one
ANOVA analysis of variance
ATF-3 activating transcription factor-3
CA cold allodynia
cAMP cyclic adenosine monophosphate
CB1 cannabinoid receptor type 1
CB2 cannabinoid receptor type 2
CBD cannabidiol
CCI chronic constriction injury
CCL2 chemokine (C-C motif) ligand 2
CD11b cluster of differentiation molecule 11B
CGRP calcitonin gene-related peptide
CIPN chemotherapy-induced peripheral neuropathy
CNS central nervous system
COX-1/2 cyclooxygenase 1 or 2
CP55,940 (-)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol
CPP conditioned place preference
DAGL diacylglycerol lipase alpha
DAGL diacylglycerol lipase beta
DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid
DRG dorsal root ganglia
ERK extracellular signal–regulated kinases
FAAH fatty acid amide hydrolase
GAT211 3-(2-Nitro-1-phenylethyl)-2-phenyl-1H-indole
GFAP  glial fibrillary acidic protein
GLAST  glutamate aspartate transporter
GLT-1  glutamate transporter 1
HU-243  (6R,8S,9S,10aR)-9-(hydroxymethyl)-6,6-dimethyl-3-(2-methyloctan-2-yl)-8,9-ditritio-7,8,10,10a-tetrahydro-6aH-benzoc[de]hromen-1-ol
IB4  isolectin B4
ICAM-1  intercellular adhesion molecule 1
IL-1β  interleukin 1 beta
IL-6  interleukin 6
JZL184  4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl)(hydroxy)methyl)piperidine-1-carboxylate
JWH015  (2-Methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone
JWH133  (6aR,10aR)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran
KML29  1,1,1,3,3,3-hexafluoropropan-2-yl 4-(bis(benzo[d][1,3]dioxol-5-yl)(hydroxy)methyl)piperidine-1-carboxylate
KT109  (4-([1,1-Biphenyl]-4-yl)-1H-1,2,3-triazol-1-yl)(2-benzylpiperidin-1-yl)methanone
KT195  [4-(4-Methoxy[1,1-biphenyl]-4-yl)-1H-1,2,3-triazol-1-yl](2-phenyl-1-piperidinyl)-methanone
LOX  lipoxygenases
LPS  lipopolysaccharide
MA  mechanical allodynia
MAGL  monoacylglycerol lipase
MAPK  mitogen-activated protein kinase
MCP-1  monocyte chemoattractant protein 1
MDA7  1-[3-benzyl-3-methyl-2,3-dihydro-1-benzofuran-6-yl]carbonyl]piperidine
MJN110  2,5-Dioxopyrrolidin-1-yl 4-[bis(4-chlorophenyl)methyl]piperazine-1-carboxylate
mRNA  messenger ribonucleic acid
MyD88  myeloid differentiation primary response gene 88
NAPE-PLD  N-acetylphosphatidylethanolamine-hydrolyzing phospholipase D
NF-κB  nuclear factor kappa-light-chain-enhancer of activated B cells
NSCLC  non-small cell lung cancer
OX42  cluster of differentiation molecule 11B/C
PGD2  prostaglandin D2
PGE2  prostaglandin E2
PGE2-G  prostaglandin E2 glycerol ester
PGF2  prostaglandin F2
Phospho-p38  phosphorylated p38 mitogen-activated protein kinase
Rimonabant  SR141716; 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide
S.E.M.  standard error of the mean
shRNA  short hairpin ribonucleic acid
SR144528  5-(4-chloro-3-methylphenyl)-1-[4-(methylphenyl)methyl]-N-[1S,2S,4R]-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1H-pyrazole-3-carboxamide
ST4070  1-biphenyl-4-ylenyl piperidine-1-carboxylate
TH  thermal (heat) hyperalgesia
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>THC</td>
<td>$\Delta 9$-tetrahydrocannabinol</td>
</tr>
<tr>
<td>TLR4</td>
<td>toll like receptor 4</td>
</tr>
<tr>
<td>TNF$\alpha$</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRPV1</td>
<td>transient receptor potential cation channel subfamily V member 1</td>
</tr>
<tr>
<td>URB597</td>
<td>(3$'$-(aminocarbonyl)[1,1$'$-biphenyl]-3-yl)-cyclohexylcarbamate</td>
</tr>
<tr>
<td>URB937</td>
<td>N-cyclohexyl-carbamic acid, 3$'$-(aminocarbonyl)-6-hydroxy[1,1$'$-biphenyl]-3-yl ester</td>
</tr>
<tr>
<td>WIN</td>
<td>WIN55,212-2; (3R)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone</td>
</tr>
</tbody>
</table>
Abstract

TARGETING MONOACYLGLYCEROL LIPASE FOR THE REVERSAL AND PREVENTION OF PACLITAXEL-INDUCED ALLODYNIA IN MICE.

By Zachary A. Curry, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2018

Major Director: Aron H. Lichtman, Ph.D., Department of Pharmacology and Toxicology

Chemotherapy-induced peripheral neuropathy (CIPN) is a side-effect of chemotherapy causing pain in the hands and feet. In particular, paclitaxel causes CIPN lasting for years without effective treatment. There is a strong need for analgesics to both treat and prevent CIPN. One system containing multiple targets to treat CIPN is the endogenous cannabinoid system. This system consists of cannabinoid type 1 (CB₁) and type 2 (CB₂) receptors primarily expressed on presynaptic neurons and cells of the immune system, respectively. Inhibition of monoacylglycerol lipase (MAGL), which hydrolyzes the endogenous cannabinoid 2-arachidonoylglycerol (2-AG), with JZL184 or MJN110 produces antinociceptive and anti-inflammatory effects in rodent pain models. In this dissertation, we test the hypothesis that
MAGL inhibitors will both reverse and prevent mouse paclitaxel-induced mechanical allodynia. JZL184 and MJN110 reversed paclitaxel allodynia in dose-dependent manners with ED$_{50}$ values (95% C.L.) of 8.4 (5.2-13.6) and 1.8 (1.0-3.3) mg/kg. Using genetic and pharmacologic approaches, we demonstrate that the anti-alldynic effects of both inhibitors require both cannabinoid receptors. As CIPN treatment could require repeated dosing, we demonstrate that repeated administration of 4 mg/kg JZL184 for six days produces anti-allodynic effects in contrast to tolerance development after repeated treatment with 40 mg/kg. We also show that MJN110 attenuates paclitaxel-induced inflammation in the spinal cord and dorsal root ganglia (DRG), namely monocyte chemoattractant protein-1 (MCP-1, CCL2) and phosphorylated p38 MAPK (phospho-p38) expression. Using the conditioned place preference (CPP) paradigm, we demonstrate that MJN110 produces a CPP in paclitaxel-treated, but not in control mice. As CIPN develops during chemotherapy, we also show that JZL184 does not interfere with the anti-proliferative and anti-apoptotic effects of paclitaxel in A549 or H460 lung cancer cell lines. Lastly, we show that co-administration of MAGL inhibitors with paclitaxel prevents the development of allodynia. Co-treatment with 5 mg/kg MJN110 or 40 mg/kg JZL184 prevents allodynia up to one or two week(s), respectively, after paclitaxel cessation. Treatment with 40 mg/kg JZL184 prevents allodynia in both CB$_1$ (+/+) and (-/-) mice, suggesting that prevention is CB$_1$-independent. Taken together, these results suggest that MAGL is a viable target for both the treatment and prevention of paclitaxel-induced allodynia in mice.
Chapter 1: Introduction

The Endogenous Cannabinoid System: Introduction

The medicinal and recreational use of Cannabis extends back as far as 2700 B.C., as documented under the reign of the Chinese Emperor Shen Nung, and includes use as an anesthetic and analgesic for surgical patients in 190 B.C. by the Chinese physician Hua T’o (Pain, 2015). In the 20th century, isolation and characterization of Δ9-tetrahydrocannabinol (THC) (Gaoni and Mechoulam, 1964) as the primary psychoactive component of cannabis, causing euphoria associated with cannabis use (Isbell et al., 1967), led to the development of synthetic cannabinoids for research use. Synthetic cannabinoids include the aminoalkylindole WIN55,212 (WIN) (Kuster et al., 1993) and CP55,940 (Fouda et al., 1987). Use of these synthetic agonists lead to the discovery of endogenous cannabinoid receptors and their corresponding endogenous ligands, which together comprise the endogenous cannabinoid system. Activation of the endogenous cannabinoid system produce antinociceptive effects in rodent (Donvito et al., 2017) models of pain, suggesting that this system of targets is useful for analgesic development.

The endogenous cannabinoid system consists of two cannabinoid receptors, two primary endogenous ligands, and enzymes regulating their synthesis and degradation. The type 1 cannabinoid (CB1) receptor (Devane et al., 1988) is found primarily on neurons and regulates
Figure 1. Overview of the endogenous cannabinoid system. This system consists of cannabinoid (CB) receptors types 1 and 2, endogenous cannabinoids 2-arachidonoylglycerol and anandamide as well as their synthetic and degradative enzymes. DAGL; diacylglycerol lipase.
NAPE-PLD; *one of several proposed biosynthetic enzymes (Blankman and Cravatt, 2013). ABHD; alpha/beta-hydrolase domain. MA GL; monoacylglycerol lipase. FAAH; fatty acid amide hydrolase. COX-1/2; cyclooxygenase 1 or 2. LOXs; lipoxygenases. TRVP1; transient receptor potential cation channel subfamily V member 1. Adapted from (Donvito et al., 2017).

nociceptive neurotransmission in the dorsal root ganglia (Hohmann and Herkenham, 1999), spinal cord (Farquhar-Smith et al., 2000) and brain (Herkenham et al., 1990). In contrast, the type 2 cannabinoid receptor (CB2) (Munro et al., 1993) is found primarily on cells of the immune system, including macrophages and lymphocytes (Munro et al., 1993), and regulates inflammation. The primary endogenous cannabinoid ligands are anandamide (AEA) (Devane et al., 1992) and 2-arachadonylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). These components of the endogenous cannabinoid system are outlined in Figure 1 and their discovery and physiologic functions are described in the following sections.

**The Endogenous Cannabinoid System: Cannabinoid Receptors**

In rodents, THC (Compton et al., 1992) or synthetic cannabinoid (Little et al., 1988) administration produces antinociceptive effects, such as increased hot water tail withdrawal latencies. Initially, THC and synthetic cannabinoids were thought to elicit psychoactive and analgesic effects through receptor-independent mechanisms such as altered cellular membrane integrity (Laurent and Roy, 1975). However, structure-activity relationships using derivatives (Handrick et al., 1982) or isomers (Martin et al., 1984) of THC suggested structural specificity required for *in vivo* effects, indicating possible receptor mediation. A radiolabeled synthetic cannabinoid CP55,940 demonstrated specific binding in rat cortical membranes (Devane et al., 1988). Comparison of the binding affinities of (-) versus (+) isomers of CP55,940 with the *in vivo* potencies of these isomers producing antinociceptive effects in the tail withdrawal test suggested that the CB1 receptor mediates the analgesic effects of CP55,940 (Devane et al., 1988;
Compton et al., 1993). In humans, the CB₁ receptor is found throughout the brain, with highest expression in the substantia nigra pars reticulata, globus pallidus (internal and external), putamen, dentate gyrus and cingulate coretex as demonstrated by CP55,940 binding (Herkenham et al., 1990). Cloning of the bovine substance K receptor 6 gene in CHO cells and activation using CP55,940 or THC confirmed the identity of the CB₁ receptor as a G-protein coupled receptor with expression in the brain overlapping that of CP55,940 binding (Matsuda et al., 1990).

Structurally, the CB₁ receptor is a G-protein coupled receptor that depresses neuronal activity through several mechanisms. Stimulation of the CB₁ receptor with direct agonists activates Gαᵢ/o proteins, decreasing cyclic AMP (cAMP) production by inhibiting adenylate cyclase (Howlett, 1984; Rhee et al., 1998). This decreases cAMP-stimulated protein kinase A activity, increasing voltage-dependent potassium currents (Hampson et al., 1995). Furthermore, the CB₁ receptor can interact with G protein-coupled inwardly-rectifying potassium (GIRK) channels (Mackie et al., 1995), promoting potassium influx into the cell to decrease neuronal excitability. CB₁ receptor activation regulates short-term synaptic plasticity by inhibiting glutamatergic (Huang et al., 2001) or GABAergic (Katona et al., 1999) signaling. The CB₁ receptor is localized to pre-synaptic termini (Hohmann and Herkenham, 1999) at multiple points in pain processing pathways. Specifically, the CB₁ receptor is expressed in the dorsal root ganglia (DRG) (Hohmann and Herkenham, 1999), spinal dorsal horn (Hohmann et al., 1999) and periaqueductal grey matter of the brain (Mailleux and Vanderhaeghen, 1992).

The cannabinoid type 2 (CB₂) receptor was discovered in 1993 when a G-protein coupled receptor with 44% sequence homology with the CB₁ receptor gene was cloned from human promyelocitic leukemia cells (Munro et al., 1993). After transfection of this gene into cultured
cells, the novel receptor expressed bound the synthetic cannabinoids WIN55,212-2 and CP55,940 with dissociation constants comparable to that generated with the CB₁ receptor (Munro et al., 1993). In contrast to the CB₁ receptor, which is highly expressed on neurons, the CB₂ receptor is primarily expressed on cells of the immune system (Munro et al., 1993). The CB₂ receptor is found on peripheral leukocytes (Bouaboula et al., 1993; Munro et al., 1993; Galiegue et al., 1995), macrophages (Munro et al., 1993), mononuclear cells (Bioque et al., 2013) and microglia (Romero-Sandoval et al., 2009a). The CB₂ receptor exhibits biased agonism as intracellular signaling from agonist binding differs between various agonists (Shoemaker et al., 2005). Depending on the particular cannabinoid agonist and concentration at the receptor site, CB₂ receptor activation increases the ERK-MAPK signaling cascade, intracellular calcium concentrations or inhibition of adenylyl cyclase (Shoemaker et al., 2005). Activation of the CB₂ receptor produces anti-inflammatory effects as CB₂ receptor direct agonists decrease pro-inflammatory cytokine (IL-1β and MCP-1) production and reverse allodynia in rodents models of neuropathic pain (Wilkerson et al., 2012a; Deng et al., 2015b). The CB₂ direct agonist JWH015 reduces macrophage chemotaxis to MCP-1 and attenuates interferon gamma-induced expression of intercellular adhesion molecule 1 (Montecucco et al., 2008). The CB₂ receptor is also up-regulated on sensory neurons following injury (Wotherspoon et al., 2005).

**The Endogenous Cannabinoid System: Anandamide and 2-arachidonoylglycerol**

In addition to the two cannabinoid receptors, there are two well-characterized endogenous ligands, AEA and 2-AG. AEA was discovered in 1992 using competition binding in rat brains, where AEA displaced the synthetic cannabinoid HU-243 from rat brain membranes and inhibited isolated smooth muscle contraction, as with synthetic cannabinoids, in a dose-dependent manner (Devane et al., 1992). The discovery of 2-AG occurred in 1995 where 2-AG
isolated from the rat brain displaced the synthetic cannabinoid CP55,940 binding to brain
membranes (Sugiura et al., 1995). 2-AG was also discovered in the canine intestines by another
group (Mechoulam et al., 1995). Isolated 2-AG from the canine gut displaced HU-243 binding
from transfected COS-7 cells expressing either CB₁ or CB₂ receptors in addition to decreasing
forkolin-induced cAMP accumulation in spleen cells similar to THC (Mechoulam et al., 1995).
When administered intravenously to mice, both 2-AG and AEA increased withdrawal latencies
in the tail withdrawal test, mimicking the effects of THC (Mechoulam et al., 1995).

Because AEA and 2-AG bind to cannabinoid receptors (Devane et al., 1992; Mechoulam
et al., 1995; Sugiura et al., 1995), they produce CB₁ and CB₂ receptor mediated effects (Kearn et
al., 1999; Hillard, 2000; Sugiura et al., 2000). However, there are key differences between AEA
and 2-AG. Both 2-AG and AEA exhibit similar selectivity for CB₁ and CB₂ receptors (Howlett et
al., 2002). However, AEA is more potent than 2-AG at the CB₁ receptor while 2-AG is more
potent than AEA at the CB₂ receptor (Howlett et al., 2002). While 2-AG acts as a full agonist at
the CB₁ receptor, AEA is only partial agonist (Sugiura et al., 1996, 2000, 2002; Hillard, 2000).
In neuroblastoma-glioma hybrid cells, application of 2-AG increased intracellular calcium levels
to those induced by the synthetic cannabinoid WIN55,212-2 (Sugiura et al., 1996). Both 2-AG
and WIN55,212-2 increased intracellular calcium levels in a CB₁-dependent manner as the CB₁
antagonist rimonabant attenuated increased intracellular calcium (Sugiura et al., 1996). However,
application of AEA to these same cells only produced a partial elevation in intracellular calcium
at high concentrations (Sugiura et al., 1996). In rat cerebellar membranes, both 2-AG and
WIN55,212-2 increased [³⁵S]GTPγS binding, a measure of CB₁ receptor activation, to
comparable levels. In contrast, AEA increased [³⁵S]GTPγS binding to about half of the
maximum levels from 2-AG or WIN55,212-2 treatment (Kearn et al., 1999; Hillard, 2000). For
the CB₂ receptor, the agonist activity for 2-AG and AEA are similar as described for the CB₁ receptor. In HL-60 cells expressing the CB₂ receptor, 2-AG increases intracellular calcium to levels comparable to those produced by the synthetic cannabinoids while high concentrations of AEA only partially increased intracellular calcium levels (Sugiura et al., 2000). Lastly, AEA can also activate vanilloid receptor 1 (TRPV1), activating nociceptive fibers exposed to high concentrations of AEA, as opposed to depressing activity in a cannabinoid receptor-dependent manner (Tognetto et al., 2001).

In addition to differences between CB₁ and CB₂ receptor activation, the levels of 2-AG and AEA differ in the brain where 2-AG levels are a thousand-fold higher than those of AEA (Sugiura et al., 1995). These brain levels are controlled by synthetic and degradative enzymes, which are further discussed in the next sections. In the brain, 2-AG regulates short-term synaptic plasticity as increased synthesis (Hashimotodani et al., 2008, 2013; Ogasawara et al., 2016) is protective against neuronal excitotoxicity in a CB₁-dependent manner (Marsicano et al., 2003). Deletion of the CB₁ receptor in principle neurons of the brain throughout the brain—including hippocampal neurons, but not cerebellar neurons or GABAergic interneurons in the hippocampus—enhances seizure-like behaviors and neuronal excitation in mice treated with kainic acid to induce seizures (Marsicano et al., 2003). Following stimulation, neurons produce 2-AG, which acts in a retrograde manner to inhibit further neurotransmitter release. This regulation of short-term synaptic plasticity is termed depolarization-induced suppression of inhibition (DSI) (Yoshida et al., 2002) or excitation (DSE) (Kreitzer and Regehr, 2001), for GABAergic and glutamatergic neurons, respectively. Both DSI and DSE are CB₁ receptor-mediated. In hippocampal pyramidal neurons, CB₁ receptor antagonists block inhibitory post-synaptic currents following depolarization or intracellular calcium release (Wilson and Nicoll, 2002).
Similarly, in cerebellar Purkinje cells, CB₁ receptor antagonists block excitatory post-synaptic currents following depolarization, reducing neurotransmitter release by decreasing calcium uptake (Kreitzer and Regehr, 2001). One form of synaptic plasticity mediated by both 2-AG (Stella et al., 1997) and AEA (Terranova et al., 1995) is the prevention of long-term potentiation in the hippocampus, which is involved in learning and memory (Nicoll, 2017).

**The Endogenous Cannabinoid System: Enzymatic Regulation of Anandamide**

The synthesis of AEA is not well-characterized. One possibility is the condensation of phosphatidylethanolamine with arachidonic acid to form N-arachidonoyl phosphatidylethanolamine (Kruszka and Gross, 1994), which is cleaved by N-arachidonoyl phosphatidylethanolamine phospholipase D (NAPE-PLD) to yield AEA (Okamoto et al., 2004; Leishman et al., 2016). However, other pathways can also synthesize AEA (Blankman and Cravatt, 2013) as NAPE-PLD (-/-) mice have brain levels of AEA comparable to (+/+), suggesting that NAPE-PLD is either not required for AEA synthesis or that alternative pathways produce AEA in NAPE-PLD (-/-) mice. In contrast to AEA synthesis, the degradation of AEA is well-characterized and primarily controlled by fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996). FAAH in an integral membrane protein that degrades anandamide in addition to other fatty acid amides, including oleamide and palmitic amide (Cravatt et al., 1996; Giang and Cravatt, 1997). FAAH is expressed in neuronal cell bodies of the neocortex, amygdala and cerebellum (Thomas et al., 1997) in addition to the spinal dorsal horn (Füllhase et al., 2016) and DRG (Lever et al., 2009).

Inhibition of FAAH with selective inhibitors, such as URB597 (Tarzia et al., 2003; Fegley et al., 2005) or PF-3845 (Ahn et al., 2009), increases AEA levels in the CNS and produces antinociceptive and anti-inflammatory effects in rodent models of pain by increasing
cannabinoid receptor stimulation (Jayamanne et al., 2006; Jhaveri et al., 2006; Kinsey et al., 2009; Naidu et al., 2010). In a mouse model of chronic constriction injury (CCI) of the sciatic nerve, mechanical or cold stimulation of the hind paw produces enhanced paw withdrawal behaviors, which is commonly described as mechanical or cold allodynia (Bennett and Xie, 1988; Le Bars et al., 2001; Kinsey et al., 2009). In mice following CCI surgery, FAAH inhibition with URB597 reverses mechanical and cold allodynia, effects attenuated by treatment with either CB$_1$ or CB$_2$ receptor antagonists (Kinsey et al., 2009). Similarly, the FAAH inhibitor PF-3845, which is more potent than URB597 and has less off-target effects (Ahn et al., 2009), reverses CCI-induced paw withdrawal to mechanical and cold stimuli in CB$_1$ and CB$_2$ (+/+ ) mice but not (-/-) mice (Kinsey et al., 2010). Both CB$_1$ expression and the anti-allodynic effects of FAAH inhibition are maintained following six day of repeated administration of PF-3845 (Schlosburg et al., 2010). In FAAH (-/-) mice, CB$_1$ expression in the brain does not differ from (+/+ ) control mice (Falenski et al., 2010). In contrast, CB$_1$ receptor expression is down-regulated in the brain with repeated administration of THC (Romero et al., 1997).

In addition to degradation by FAAH, AEA can also undergo cyclooxygenation by COX-2 to produce prostaglandin-ethanolamides (Yu et al., 1997; Kozak et al., 2002; Gatta et al., 2012). In a mouse model of inflammatory knee pain, the levels of prostamide F2$\alpha$ was elevated without changes in 2-AG or AEA levels (Gatta et al., 2012). Inhibition of COX-1 or COX-2 significantly reduced spinal prostamide F2$\alpha$ levels (Gatta et al., 2012). In naïve mice, intrathecal administration of prostamide F2$\alpha$ increased activity of spinal nociceptive neurons and, in the whole animal, lowered paw withdrawal latencies to a heat stimulus (Gatta et al., 2012). These effects were blocked by a prostamide F2$\alpha$ receptor antagonist (Gatta et al., 2012). In mice with inflammatory knee pain, intrathecal administration of a prostamide F2$\alpha$ receptor antagonist
reduced nociceptive neurotransmission and increased paw withdrawal latencies (Gatta et al., 2012). Taken together, these results suggest that prostamide F2α plays a role in nociceptive neurotransmission in inflammatory knee pain. However, it is unknown whether prostamide F2α or related prostaglandin ethanolamides contribute to allost dysnia development or maintenance in models of neuropathic pain.

**The Endogenous Cannabinoid System: Enzymatic Regulation of 2-arachidonoylglycerol**

Synthesis of 2-AG is well-characterized and involves calcium-dependent phospholipase C degradation of membrane phospholipids into diacylglycerols with subsequent cleavage by diacylglycerol lipase (DAGL) enzymes to produce 2-AG (Stella et al., 1997; Piomelli, 2003). There are two subtypes of DAGL enzymes: alpha (DAGLα) and beta (DAGLβ) (Bisogno et al., 2003). While DAGLα is highly expressed in neurons (Bisogno et al., 2003), DAGLβ is found in macrophages and microglia (Hsu et al., 2012). The degradation of 2-AG is controlled by several enzymes, including MAGL (Dinh et al., 2002), alpha/beta-hydrolase domain (ABHD) containing 12, ABHD6, and FAAH (Blankman et al., 2007). However, MAGL is responsible for over 80% of 2-AG hydrolysis in the brain, followed by ABHD12 (<10%), ABHD6 (<5%) and FAAH (<2.5%) as determined by activity-based protein profiling (Blankman et al., 2007). In presynaptic neurons of the brain (Dinh et al., 2002) and spinal dorsal horn (Horváth et al., 2014), MAGL is a soluble protein associated with the cytosolic side of the cellular membrane (Blankman et al., 2007) where it functions as a serine hydrolase (Karlsson et al., 1997) degrading 2-AG. In the brain, MAGL regulates the length of depolarization-induced suppression of excitation, where expression in the hippocampus decreases 2-AG levels, decreasing CB1 receptor stimulation (Straiker et al., 2009).
By hydrolyzing 2-AG into arachidonic acid, MAGL regulates the levels of arachidonic acid in the central nervous system both under basal conditions and during neuroinflammation (Nomura et al., 2011b). In the brain, inhibition of MAGL decreases basal levels of arachidonic acid levels, derivative pro-inflammatory prostaglandins (PGE$_2$, PGD$_2$, PGF$_2$), and thromboxane B2 (Nomura et al., 2011b). Following LPS challenge, which increases synthesis of arachidonic acid and its metabolites, MAGL inhibition decreases these lipids and attenuates inflammatory signaling as evidenced by decreased pro-inflammatory cytokine (IL-1β, IL-6, TNFα) production (Nomura et al., 2011b). The production of pro-inflammatory prostaglandins in the brain appears to be astrocyte-dependent and involves transport of 2-AG and arachidonic acid between neurons and astrocytes to regulate 2-AG metabolism (Viader et al., 2015). As arachidonic acid metabolites, including prostanoids (Zeilhofer, 2007) and leukotrienes (Noguchi and Okubo, 2011) regulate nociception and inflammation through multiple signaling cascades, MAGL plays a critical role in controlling inflammation in the CNS. While MAGL also controls LPS-induced arachidonic acid and prostaglandin synthesis in the liver and lung, calcium-dependent phospholipase A2 controlled synthesis of these metabolites in the spleen and gut.

Inhibition of MAGL produces both anti-allodynic and anti-inflammatory effects in rodent models of pain by (1) increasing cannabinoid receptor activation with increased 2-AG levels and (2) decreasing arachidonic acid levels and subsequent metabolism (Long et al., 2009a). Similarly to FAAH inhibition, inhibition of MAGL with JZL184 (Long et al., 2009a) or MJN110 (Niphakis et al., 2013) reverses CCI-induced bilateral mechanical allodynia, cold allodynia and heat hyperalgesia produced by three chromic gut sutures through CB$_1$- and CB$_2$-mediated mechanisms of action (Ignatowska-Jankowska et al., 2015). However, in CCI models producing unilateral mechanical and cold allodynia through the use of silk (Kinsey et al., 2010) or two
chromic gut (Kinsey et al., 2009) sutures, reversal of mechanical allodynia by JZL184 is CB₁, but not CB₂, -dependent. In the carrageenan model of inflammatory pain, JZL184 reduces mechanical alldynia through CB₁- and CB₂-mediated mechanisms of action and paw edema in a CB₂-dependent manner (Ghosh et al., 2013).

While suppressing enhanced responses to mechanical, thermal or cold stimuli, repeated administration of MAGL inhibitors can be limited by the development of tolerance to their anti-allodynic effects. Repeated administration of high-dose (40 mg/kg, i.p.) JZL184 for six days causes loss of anti-allodynic effects in the CCI model of neuropathy and cross-tolerance to the anti-allodynic effects of PF-3845 and WIN55,212-2 (Schlosburg et al., 2010). Similarly, constitutive MAGL deletion in mice attenuates the antinociceptive effects of JZL184 and THC (Schlosburg et al., 2010). These effect correspond to decreased CB₁ receptor expression and function in the cingulate cortex, somatosensory cortex, hippocampus and periaqueductal grey following repeated administration of JZL184 (Schlosburg et al., 2010). Decreased CB₁ expression and function is likely due to higher levels of 2-AG in the whole brain observed with repeated administration of high-dose JZL184 compared to acute treatment (Kinsey et al., 2013). The development of tolerance to the anti-allodynic effects of JZL184 and down-regulation of CB₁ receptor expression and function is not observed with a repeated administration of a low dose of JZL184, 4 mg/kg, which causes a more gradual increase in 2-AG levels (Kinsey et al., 2013). Therefore, a low dose treatment strategy avoids the development of tolerance to MAGL inhibition after six days of repeated administration.

As with repeated administration of a low-dose of JZL1184, there are other strategies to circumvent tolerance to MAGL inhibition. These include combining FAAH and MAGL inhibitors, which retain anti-allodynic effects without CB₁ receptor desensitization in the
carrageenan model of inflammatory pain (Ghosh et al., 2015), and combinations of MAGL inhibitors with other analgesics. In mice following CCI surgery, MJN110 synergizes with morphine to reverse mechanical allodynia without the development of tolerance to repeated administration of MJN110 and morphine in combination (Wilkerson et al., 2016b). Similarly, combined treatment of gabapentin with the MAGL inhibitor KML29 additively reduced mechanical allodynia and synergistically reduced cold allodynia in mice following CCI surgery (Crowe et al., 2017). Combined treatment with gabapentin and KML29 did not cause tolerance development in mechanical allodynia with repeated administration, but partial tolerance in cold allodynia was observed (Crowe et al., 2017). Likewise, in the paclitaxel model of CIPN, a CB1 positive allosteric modulator, GAT211, synergizes with JZL184 to reverse mechanical allodynia without the development of tolerance to repeated treatment (Slivicki et al., 2017).

In addition to metabolism by MAGL, 2-AG can also undergo cyclooxygenation by COX-2 to produce prostaglandin glycerol esters including prostaglandin E2 glycerol ester (PGE2-G) (Kozak et al., 2000, 2002). In murine macrophage-like RAW264.7 cells, treatment with PGE2-G decreased LPS-induced NF-κβ activation similar to PGE2 (Hu et al., 2008). While PGE2-G levels are decreased in the hindpaw of rats treated with either the MAGL inhibitor URB602 or the COX-2 inhibitor nimesulide, PGE2-G is quickly metabolized and receptors mediating the effects of PGE2-G have not yet been identified (Hu et al., 2008). In the carrageenan model of inflammatory pain, PGE2 levels were increased in the hindpaw while PGE2-G levels were unchanged by carrageenan-induced inflammation (Hu et al., 2008). While metabolism of 2-AG into prostaglandin-glycerol esters may have anti-nociceptive or anti-inflammatory effects, the roles of prostaglandin glycerol esters are not yet clear. One way to further examine the effects of prostaglandin glycerol esters in these models is to use substrate-selective inhibitors of COX-2,
which prevent metabolism of 2-AG while sparing prostaglandin production from arachidonic acid (Hermanson et al., 2013, 2014). These experiments also need to be repeated using a more potent MAGL inhibitor targeting the active site, such as MJN110 (IC$_{50} = 2.1$nM) (Niphakis et al., 2013), as URB602 is less potent (IC$_{50} = 28$ µM) and inhibits MAGL in a non-competitive manner (Hohmann et al., 2005). It is unknown whether prostaglandin glycerol esters play a role in the development and maintenance of allodynia in models of neuropathic pain.

**The Endogenous Cannabinoid System: Anti-Cancer Effects**

In addition to antinociceptive effects in pre-clinical models of neuropathy, modulation of the endogenous cannabinoid system has anti-tumor effects in numerous cancer types, including cancers of the breast, lung and ovary (Ramer and Hinz, 2017). As lung and bronchus cancers are expected to account for the majority of cancer deaths in American men (26%) and women (25%) in 2018 (Siegel et al., 2017), pharmacologic modulation of the endogenous cannabinoid system may provide new chemotherapeutic strategies. Notably, THC inhibits cell motility, and invasion in the A549 and SW-1573 cell lines of non-small cell lung cancer (Preet et al., 2008). In both A549 and SW-1573 cell lines of non-small cell lung cancer, application of the mixed CB$_1$/CB$_2$ receptor agonist WIN55,212-2 or the direct CB$_2$ receptor agonist JWH015 attenuated cell proliferation and migration in response to epidermal growth factor (Preet et al., 2011). Inhibition of migration by WIN55,212-2 required both CB$_1$ and CB$_2$ receptors, as evidenced by co-treatment with CB$_1$ or CB$_2$ antagonists (Preet et al., 2011). Likewise, reduced cell migration by JWH015 was CB$_2$-dependent (Preet et al., 2011). When A549 cells were implanted subcutaneously in nude mice, systemic administration of WIN55,212-2 reduced tumor volumes relative to control animals, an effect that was both CB$_1$ and CB$_2$-mediated (Preet et al., 2011). Similarly, systemic administration of the CB$_2$ receptor agonist JWH133 reduced tumor growth in
a CB2-dependent manner (Preet et al., 2011). Post-mortem microscopy of these tumors suggests that WIN55,212-2 and JWH133 reduced A549 tumor volumes by decreasing angiogenesis and increasing apoptosis (Preet et al., 2011).

In addition to direct agonists targeting the cannabinoid receptors, modulation of AEA or 2-AG levels may have chemotherapeutic effects (Ramer and Hinz, 2017). Inhibition of FAAH with URB597 or knockdown of FAAH with siRNA in A549 cells decreases cellular invasion as measured using matrigel (Winkler et al., 2016). Reduced invasion secondary to FAAH inhibition is CB2-mediated as co-treatment with a CB2 receptor antagonist, but not a CB1 antagonist, restored the invasive phenotype (Winkler et al., 2016). Following intravenous administration of A549 cells in nude mice, four weeks of URB597 treatment (1-10 mg/kg, i.p. every 72 h) caused a significant reduction in metastatic lung nodules in a dose-dependent manner (Winkler et al., 2016). Similarly, treatment with AEA or 2-AG (10 mg/kg, i.p. on day 1, followed by 5 mg/kg every 72 h) for four weeks in nude mice with A549 cells significantly reduced metastatic nodules in the lungs (Winkler et al., 2016). These results suggest that both endogenous cannabinoids have chemotherapeutic properties; however, MAGL inhibitors have not been evaluated in vitro or in vivo in cell lines of non-small cell lung cancer.

MAGL plays a significant role in melanoma, ovarian and breast cancers with an “aggressive” phenotype indicating poor survival and increased metastatic burden (Arpino et al., 2015) linked to MAGL expression (Nomura et al., 2010). In aggressive versus non-aggressive melanoma, ovarian and breast cancer cells, MAGL expression is increased along with decreased levels of monoacylglycerols and increased free fatty acid levels (Nomura et al., 2010). These results extend to human primary tumors, where high-grade ovarian tumors demonstrate increased MAGL activity and free fatty acids compared to low-grade (benign) tumors (Nomura et al.,
2010). Inhibition of MAGL with JZL184 (1 μM for four hours) or knockdown with shRNA in cancer cells with an aggressive phenotype elevated monoacylglycerols and decreased free fatty acids to levels comparable to non-aggressive cancer cells (Nomura et al., 2010). Knockdown of MAGL in aggressive melanoma, ovarian and breast cancer cells decreased cell migration, invasion and survival (Nomura et al., 2010). In nude mice with subcutaneous xenographs, daily JZL184 administration (40 mg/kg, oral) or knockdown of MAGL decreased tumor volume compared to control during 30 days following implantation (Nomura et al., 2010). In non-aggressive MUM2C melanoma cells, overexpression of MAGL increased cellular free fatty acids, cell migration into matrigel and, when implanted into nude mice, tumor growth comparable to cancer cells with the aggressive phenotype (Nomura et al., 2010). Treatment with JZL184 decreased free fatty acid levels and cell migration into matrigel (Nomura et al., 2010). Application of free fatty acids, palmitic acid or stearic acid, attenuated these effects of JZL184 in vitro (Nomura et al., 2010). To demonstrate the link between free fatty acids and enhanced tumor aggression, nude mice were subcutaneously implanted with aggressive melanoma cells with MAGL stably knocked down (Nomura et al., 2010). While cells lacking MAGL expression exhibited reduced tumor growth, tumor volumes were significantly increased in mice on a high-fat diet (Nomura et al., 2010). Taken together, these results suggest that MAGL inhibition regulates free fatty acid levels associated with an aggressive phenotype (Nomura et al., 2010). Lipidomic analysis of cells with MAGL overexpression or knockdown suggests that the aggressive phenotype is mediated by decreased levels of monoacylglycerols and increased conversion into free fatty acids and their secondary metabolites, including phospholipids and lysophospholipids (Nomura et al., 2010). Taken together, these findings suggest that MAGL regulates breast, ovarian and melanoma cancer aggressiveness by increasing fatty acid
production, which is cannabinoid receptor-independent. In an extension of this study in prostate cancer cells, JZL184 reduced cell migration *in vitro*, an effect that was partially attenuated by co-treatment with the CB₁ receptor antagonist rimonabant (Nomura et al., 2011a). Therefore, depending on the cancer type and cannabinoid receptor expression, MAGL regulates lipid signaling contributing to cannabinoid receptor-dependent and -independent mechanisms of cancer invasion and metastasis.

**The Endogenous Cannabinoid System: Current Therapeutic Indications and Potential Analgesic Use**

Historical use of cannabis includes use as a surgical analgesic in ancient China (Pain, 2015) and written reports of cannabis use to treat inflammatory and “neurologic” pain in the 19th century (Mikuriya, 1969). Today, the legalization of recreational marijuana and medical marijuana use in the United States has renewed interest in the therapeutic use of cannabis. As of 2018, the United States Food and Drug Administration has approved two cannabinoid-based drugs for therapeutic use: (1) Nabilone, a THC derivative, for the treatment of chemotherapy-induced nausea and vomiting and (2) Dronabinol, synthetic THC, for the treatment of chemotherapy-induced nausea as well as AIDS-related anorexia and weight loss (Whiting et al., 2015). While not approved in the United States, the cannabis extract nabiximols is approved in Canada for the treatment of cancer pain as well as spasticity from multiple sclerosis (PDQ® Integrative, Alternative, 2017). In 2015, a meta-analysis summarized the results of 79 randomized controlled trials examining the use of cannabinoid-based drugs (primarily cannabis extracts or THC derivatives) for the management of various medical conditions, including chemotherapy-induced nausea and vomiting and chronic pain (Whiting et al., 2015). For chemotherapy-induced nausea and vomiting, cannabinoid-based therapies produced a greater
response than placebo with an odds ratio of 3.82 (95% CI, 1.55-9.42) (Whiting et al., 2015). For chronic pain, cannabinoid-based therapies were evaluated for the treatment of pain from various mechanisms, including neuropathic pain, cancer pain, and diabetic peripheral neuropathy. Overall, cannabinoid-based therapies produced a greater response than placebo with an odds ratio of 1.41 (95% CI, 0.99-2.00) (Whiting et al., 2015). In a separate meta-analysis, examination of inhaled cannabis for the treatment of chronic neuropathic pain demonstrated that inhaled cannabis produced a greater response than placebo with an odds ratio of 3.22 (95% CI, 1.59-7.24) (Andreae et al., 2015). This study included chronic pain syndromes that remain difficult to treat such as HIV-related sensory neuropathy, neuropathic pain secondary to trauma, spinal cord pain and mixed peripheral neuropathies (Andreae et al., 2015). However, not all studies examining cannabinoids as for the treatment of neuropathic pain support clinical use. In another meta-analysis examining multiple analgesics for the treatment of neuropathic pain, nine trials examining cannabinoid-based therapies for the treatment of neuropathic pain did not demonstrate substantial benefit compared to placebo (Finnerup et al., 2015).

In addition to neuropathic pain, cannabinoid-based therapies also need to be investigated for other types of neuropathies, including chemotherapy-induced peripheral neuropathy. In a crossover pilot study examining chemotherapy-induced peripheral neuropathy in 16 patients, treatment with nabiximols produced no significant differences compared with placebo control (Lynch et al., 2014). While this study was underpowered, 5 of the 16 patients (about 31%) reported a significant reduction in pain scores as demonstrated by responder analysis (Lynch et al., 2014). Based on this pilot study, a randomized clinical trial with at least 183 patients would be needed for comparison of nabiximols versus placebo for this type of neuropathy (Lynch et al., 2014). In a larger double-blind, placebo-controlled clinical trial examining nabiximols for the
treatment of non-neuropathic cancer pain, pain scores were not significantly reduced by drug treatment (p=0.0854) (Lichtman et al., 2017). Secondary endpoints, including sleep duration and patient satisfaction score demonstrated significant improvement over placebo, suggesting that nabiximols could be of benefit for patients with cancer pain (Lichtman et al., 2017).

While cannabinoids could be used to treat chronic pain, the use of medical marijuana or other cannabinoid-based therapies must be balanced with undesirable side-effects (Hill, 2015; Whiting et al., 2015). In randomized controlled trials, the likelihood of adverse events due to cannabinoid-based therapy use was higher than placebo with an odds ratio of 3.03 (95% CI, 2.42-3.80) (Whiting et al., 2015). The most common adverse events reported were psychoactive effects including dizziness, disorientation, confusion and euphoria (Whiting et al., 2015). Taken together, these results suggest that cannabinoid-based therapeutics can be used to treat neuropathic pain; however, psychoactive side effects are of concern. Therefore, novel therapeutic strategies, such as modulation of the endogenous cannabinoid system, are needed to treat neuropathic pain without the development of psychoactive side effects associated with cannabis use.

**Chemotherapy-Induced Peripheral Neuropathies: A Clinical Problem**

Chemotherapy-induced peripheral neuropathies (CIPN) are a dose-limiting side-effect of various chemotherapeutics, including paclitaxel, cisplatin and vincristine that remain difficult to treat (Loprinzi, 2017). As evidenced by pre-clinical rodent models of CIPN, discussed later in this dissertation, modulation of the endogenous cannabinoid system may be a useful therapeutic strategy. Here, we will introduce CIPN in both humans and animal models. Next, we propose a rationale for the focus on paclitaxel CIPN and describe the mechanisms underlying neuropathy development.
Advances in cancer research over the last several decades have identified a variety of pharmacologic targets aimed at the “hallmarks of cancer” which include evasion of apoptosis, self-sufficiency in growth signals, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). While cancer survivorship is increasing, with 15.5 million cancer survivors in the United States in 2016 versus 3.6 million in 1975 (Bluethmann et al., 2016), many chemotherapeutic agents lead to the development of long-term side-effects warranting consideration prior to initiation of chemotherapy (Ahmad et al., 2016). Of particular concern is the development of chemotherapy-induced peripheral neuropathies (CIPN). CIPN is associated with a variety of anti-neoplastic agents, including paclitaxel, cisplatin and vincristine, and can occur in as many as 68% of patients receiving chemotherapy (Seretny et al., 2014). Despite risks of use, these chemotherapeutics are widely prescribed and are included in the World Health Organization’s List of Essential Medications for a basic healthcare system (WHO, 2017). Indications for paclitaxel, cisplatin or vincristine use are broad and include ovarian, early and metastatic breast, non-small cell lung, lymphoma and nasopharyngeal cancers (WHO, 2017).

The presentation of CIPN varies depending on the chemotherapeutic used, dose and timing of chemotherapy. Other factors that could modify CIPN presentation include patient race and diagnosed comorbid conditions, including diabetic neuropathy (Bhatnagar et al., 2014; Hershman et al., 2016). Patients receiving paclitaxel chemotherapy typically present with a sensory neuropathy in a “stocking and glove” distribution including the hands and feet (Dougherty et al., 2004). Along with pain, cold, tingling or numbness in these areas, patients report impaired sensitivity to light mechanical touch and detection of sharp stimuli, suggesting impairment of Aβ and Aδ fiber signaling (Dougherty et al., 2004). In addition to symptoms of neuropathy, paclitaxel treatment can cause myelosuppression (neutropenia, which can be life-
threatening), myalgia and alopecia (Onetto et al., 1993; Ghersi et al., 2015; Loprinzi, 2017). For patients receiving cisplatin, neuropathy development is similar to paclitaxel and follows a “stocking and glove” distribution (Kedar et al., 1978; Hadley and Herr, 1979); however, nephrotoxicity secondary to decreased glomerular filtration rate is a significant concern (Stark and Howell, 1978) in addition to ototoxicity (Al-Khatib et al., 2010), nausea and vomiting (Piel and Perlia, 1975). For vincristine CIPN, sensory abnormalities are primarily in the lower limbs and can progress to motor dysfunction, including foot drop and loss of tendon reflexes (Lavoie Smith et al., 2015; Loprinzi, 2017).

For patients experiencing CIPN, resolution of neuropathy symptoms following chemotherapy cessation varies by chemotherapy type (Loprinzi, 2017). For paclitaxel CIPN, the median duration of CIPN symptoms is approximately two years with neuropathy persisting in as many as 64% of patients at one year and 41% of patients three years after treatment (Tanabe et al., 2013). For patients receiving cisplatin, symptoms worsen after cessation of chemotherapy in 31% of patients (Siegal and Halm, 1990), but can rapidly resolve. In one study, 65% of patients experienced cisplatin CIPN three months after chemotherapy cessation which decreased to 17% at one year post-treatment (Von Schlippe et al., 2001). Vincristine CIPN also worsens following cessation of treatment (Verstappen et al., 2005) and occurs in up to 92% of patients receiving vincristine (Haim et al., 1994). However, vincristine neuropathy resolution is faster than for cisplatin with a median duration of three months (Haim et al., 1994).

Despite the duration of CIPN symptoms, there are no effective treatments for chemotherapy neuropathies. As with other types of neuropathies and neuropathic pain, non-steroidal anti-inflammatory drugs and opioids are not clinically indicated (Kim et al., 2015). Trials of agents used to treat other types of neuropathic pain, anti-depressants such as
amitriptyline (Kautio et al., 2009) and venlafaxine (Zimmerman et al., 2016) and anticonvulsants, such as pregabalin (Pfizer, 2008), fail to relieve CIPN symptoms. Although the antidepressant duloxetine, a serotonin-norepinephrine reuptake inhibitor, can be used to treat CIPN, it has modest efficacy for cisplatin neuropathies, but is of no benefit over placebo for taxane-induced CIPN, including paclitaxel CIPN (Smith et al., 2013). The efficacy of duloxetine for vincristine neuropathies is inconclusive (Hirayama et al., 2015) and needs further study.

Due to the lack of efficacious treatments for the persistent symptoms of CIPN, reduction or cessation of the chemotherapy use remains the standard of care for CIPN management and is typically reserved for patients with severe neuropathy symptoms or significant functional impacts due to CIPN (Loprinzi, 2017), such as inability to work (Zanville et al., 2016). In patients requiring a paclitaxel dose reduction due to side-effects of treatment, 17% were specifically due to CIPN symptoms (Bhatnagar et al., 2014). In particular, patients of African American descent are at increased risk for a dose reduction due to CIPN symptoms (Bhatnagar et al., 2014), which could be due to a higher prevalence of diabetes and diabetic peripheral neuropathy (Hershman et al., 2016). As deviation from established antineoplastic protocols could hamper survival, there is a serious need to identify novel analgesics capable of treating chemotherapy-induced neuropathic pain.

**Paclitaxel, Cisplatin and Vincristine: Chemotherapeutic Mechanisms of Action**

In order to develop therapeutic strategies for CIPN management, the mechanisms of neurotoxicity and neuropathy development are being explored using pre-clinical models. Paclitaxel, cisplatin and vincristine are cytotoxic through distinct mechanisms. These mechanisms contribute to neuropathy development due to off-target effects in cells of the nervous system.
In cells, paclitaxel causes microtubule stabilization (Schiff and Horwitz, 1980) by binding to the β-subunit of tubulin, stabilizing interactions between tubulin protofilaments forming microtubules (Nogales et al., 1995; Snyder et al., 2001). Paclitaxel initiates cell death by preventing the formation of the mitotic spindle, arresting progression through mitosis (De Brabander et al., 1981). The exact mechanism of paclitaxel-induced cancer cell death is unclear and appears to be caspase-independent despite activation of apoptosis (Huisman et al., 2002). Similar to paclitaxel, vincristine exhibits cytotoxicity by altering microtubule dynamics. While paclitaxel stabilizes microtubule polymerization, vincristine destabilizes microtubule polymerization by binding to the tubulin subunits (Owellen et al., 1972) causing loss of mitotic spindle function and metaphase arrest (Jordan et al., 1991). In contrast to chemotherapeutic agents causing disruption of microtubule homeostasis, cisplatin exhibits anti-tumor effects by binding to cytosine and guanine in nuclear DNA (Stone et al., 1974). In cancer cells, cisplatin causes growth arrest throughout the cell cycle (Bergerat et al., 1979). While the arrest of cell growth with these chemotherapeutics is beneficial for cancer chemotherapy, other effects of these agents on sensory neurons remain problematic. Because neuronal alterations and inflammation from chemotherapy involve sensory pathways that cannot be replicated in vitro, whole animal models are useful for the assessment of both the sensory and affective/motivational aspects of chemotherapy-induced neuropathic pain.

**Animal Models of CIPN: Behavioral Changes to Mechanical, Thermal and Cold Stimuli**

In rat and mouse models of CIPN, administration of chemotherapeutics produces behavioral changes to hind paw stimulation, mirroring the “stocking and glove” distribution of sensory symptoms in humans (Dougherty et al., 2004; Loprinzi, 2017). In rodents, administration of paclitaxel produces mechanical allodynia (enhanced withdrawal) to light hind paw stimulation
Polomano et al., 2001). In addition to hyper-reflexive responses to mechanical stimuli, paclitaxel elicits heat hyperalgesia and cold allodynia with enhanced paw withdrawal responses to hot and cold stimuli, respectively, in the absence of motor impairment (Polomano et al., 2001). Comparisons between different mice strains, such as C57BL/6J versus DBA/2J, show greater mechanical allodynia development in DBA/2J mice compared to C57BL/6J mice while the magnitude of cold allodynia was comparable between the two strains and neither developed heat hyperalgesia (Smith et al., 2004). This suggests that the behavioral changes following identical schedules of paclitaxel administration have a strong genetic component.

Similar to paclitaxel treatment, cisplatin administration in rodents produces a mechanical allodynia as demonstrated by paw withdrawal (Authier et al., 2000, 2003). Cisplatin administration also produces cold allodynia (Authier et al., 2003) and heat hyperalgesia (Cata et al., 2008). However, cisplatin-induced nephrotoxicity, which is a significant clinical concern (Stark and Howell, 1978), can also cause mortality in rodent models. Blood creatinine and ketones are significantly increased with decreases in body weight and temperature following cisplatin administration to rats (Guindon and Hohmann, 2013); however, these effects can be prevented by treatment with sodium bicarbonate prior to cisplatin without changing the magnitude of mechanical alldynia (Guindon and Hohmann, 2013).

As with rodent models of paclitaxel and cisplatin CIPN, administration of vincristine to rodents also includes a mechanical and heat hypersensitivity to hind paw stimulation without changes in motor function (Aley et al., 1996). However, heat hyperalgesia was only tested on the last day of treatment (Aley et al., 1996). In a separate study examining paw withdrawal to a heat stimulus over time, vincristine administration produced significant increases in withdrawal latencies compared to control, suggesting hypoalgesia (Authier et al., 1999). As with cisplatin,
vincristine administration to animals produces dose-dependent toxic effects, including dyspnea with changes in respiratory sounds with increasing doses (Nozaki-Taguchi et al., 2001). Using these rodent models, the development of CIPN neuropathies has been characterized in order to identify behavioral changes related to neuropathy, mechanisms of neuropathy and targets for therapeutic development.

**Changes in Affective Behaviors from CIPN**

In addition to traditional measures of pain in pre-clinical animal models, which utilized depressed stimulus-evoked behaviors as a measure of analgesia, pain is associated with the development of an aversive state and a decrease in functional behaviors. Patients experiencing CIPN report functional impacts, including decreased ability to work (Zanville et al., 2016), depression, anxiety and decreased quality of sleep (Hong et al., 2014) due to symptoms of neuropathy. Similarly, other conditions producing chronic pain are comorbid with functional impacts, including depression (Stubbs et al., 2017). In models of paclitaxel CIPN, the development of an anxiety or depression-like phenotype is also considered. In C57BL/6J mice, administration of paclitaxel produces both mechanical and cold allodynia (Toma et al., 2017). Paclitaxel treatment in these mice also led to a preference for the dark chamber in the light/dark box assay and immobility in the forced swim test (Toma et al., 2017). Mechanical allodynia was present for 1 through 11 weeks after paclitaxel treatment while cold allodynia was only tested up to three weeks post-paclitaxel (Toma et al., 2017). While alterations in the light/dark box were observed at all time points assessed, including 2-3 weeks and 6-7 weeks post-paclitaxel, alterations in the forced swim test were only noted 2-3 weeks post-paclitaxel and not 1 week or 4-5 weeks after paclitaxel (Toma et al., 2017). In rodents, preference for the dark chamber is abolished by treatment with a benzodiazepine (Belzung et al., 1987). Immobility in the forced
swim test is suppressed by treatment with tricyclic antidepressants (Porsolt et al., 1978; Hu et al., 2009). However, for paclitaxel-treated mice, it is unknown if these observations in mice are the result of neuropathy development or an alternate effect of paclitaxel treatment.

The conditioned place preference (CPP) paradigm is increasingly used as a screening tool for candidate analgesics to incorporate the affective and sensory components of pain relief (Navratilova and Porreca, 2014). The conditioned place preference paradigm uses classical conditioning to pair drug treatment with a particular chamber of an apparatus with three distinct chambers: two to be paired with drug or vehicle treatment and a central acclimation chamber. Each chamber has distinguishing characteristics such as wall color or flooring. Rodents are treated with a drug (unconditioned stimulus), such as one with rewarding effects (unconditioned reward), and allowed to acclimate in one of the chambers (conditioned stimulus) without access to the other chambers. The next day, the rodent is treated with the vehicle for drug treatment and acclimated to the opposite chamber. Vehicle or drug treatment is given on alternate days followed by acclimation to the appropriate chamber. On the final test day, animals are placed in the central chamber and allowed to explore the whole apparatus in a drug-free state. An increase in time spent in the drug-paired chamber versus the control chamber is considered a conditioned place preference (CPP), the conditioned response. Alternatively, a decrease in time spent in the drug-paired chamber relative to control is considered a conditioned place aversion (CPA) (Prus et al., 2009).

Using this model, rodents with a surgical, neuropathic (e.g., CIPN) or inflammatory model of nociception are treated with a candidate analgesic and conditioned to one of two chambers. The other chamber is paired with placebo (vehicle) treatment. When allowed to explore the apparatus in the drug-free state, an increase in the amount of time spent in the drug-
paired chamber is interpreted as analgesia or reward from pain relief (Navratilova and Porreca, 2014). This approach has been successfully applied to a variety of models of chronic pain, including spinal nerve ligation (King et al., 2009), bone cancer pain (Remeniuk et al., 2015) and osteoarthritis (Havelin et al., 2016).

Using this approach, candidate analgesics have been tested in models of cisplatin-induced neuropathies. In cisplatin-treated mice displaying mechanical allodynia, gabapentin treatment produced a CPP for the drug-paired chamber while ketorolac treatment did not (Park et al., 2013). While gabapentin and NSAIDS are not recommended for the treatment of CIPN (Kim et al., 2015; Loprinzi, 2017), gabapentin is recommended for the treatment of other types of neuropathic pain (Finnerup et al., 2015). Control animals lacking cisplatin did not display a preference for the gabapentin-associated chamber over saline control (Park et al., 2013). For cisplatin CIPN, the development of a CPP for a drug-paired chamber is interpreted as relief from tonic pain from neuropathy symptoms with the lack of preference in control animals suggesting that a drug lacks intrinsic rewarding effects (Park et al., 2013). A similar approach was used to screen a histone deacetylase inhibitor as a potential analgesic for mice treated with cisplatin (Krukowski et al., 2017). Following conditioning, cisplatin-treated mice demonstrated a CPP for the drug-paired chamber versus the vehicle control chamber. In contrast, control mice lacking cisplatin spent an equal amount of time in each chamber (Krukowski et al., 2017). These results were interpreted as relief from spontaneous pain due to cisplatin CIPN (Krukowski et al., 2017).

For paclitaxel neuropathies, only morphine treatment has been tested in the CPP paradigm (Neelakantan et al., 2016). The dose-response relationship for the development of a CPP with morphine treatment did not differ between paclitaxel-treated or control mice when tested ten days after paclitaxel cessation (Neelakantan et al., 2016). This is consistent with clinical findings
where opioids are not recommended for the treatment of CIPN (Kim et al., 2015; Loprinzi, 2017).

**Rationale for Focus on Paclitaxel CIPN**

As paclitaxel CIPN affects as many as 41% of patients three years after treatment (Tanabe et al., 2013) while cisplatin (Von Schlippe et al., 2001) and vincristine (Haim et al., 1994) CIPN symptoms resolve in the majority of patients within one year, there is a need for therapeutic strategies to treat and prevent paclitaxel CIPN. Therefore, this thesis emphasizes mechanisms underlying paclitaxel-specific neuropathy in animal models. The development of paclitaxel neuropathies involve concurrent effects at distinct anatomic locations with a general pattern of peripheral neuronal damage followed by inflammatory changes in both the dorsal root ganglia (DRG) and spinal dorsal horn (Peters et al., 2007). These changes and their associated biomarkers are summarized in figure 2 and described in detail in the following sections.

**Figure 2. Markers of paclitaxel-induced allodynia by anatomic location.** In the periphery, paclitaxel causes nerve fiber loss through direct damage of peripheral neurons (Bobylev et al., 2015). In the dorsal root ganglia, paclitaxel activates toll-like receptor 4 (TLR4) (Li et al., 2014),
causing downstream phosphorylation of p38 (phospho-p38) (Li et al., 2015) in addition to monocyte chemoattractant protein 1 (MCP-1) transcription and release (dashed arrow) (Zhang et al., 2013). MCP-1 release is TLR4-dependent (Zhang et al., 2016a) and could be via NF-κB activation (Byrd-Leifer et al., 2001; Thompson and Van Eldik, 2009). In the spinal dorsal horn, paclitaxel causes MCP-1 release from astrocytes (Zhang et al., 2013). Mechanisms of inflammation and neuropathy due to MCP-1 and phospho-p38 expression are also noted and are further described in the text.

**Paclitaxel CIPN: Peripheral Toxicity**

In the periphery, paclitaxel impairs neuronal mitochondrial function and transport as a secondary consequence of microtubule stabilization. While microtubule stabilization by paclitaxel halts tumor cell progression through mitosis (Schiff and Horwitz, 1980; De Brabander et al., 1981), homeostatic microtubule function in neurons is also altered. In cultured neurites, paclitaxel treatment significantly reduced the distance and velocity of anterograde mitochondrial transport from the cell body to peripheral processes, an effect causing organelle accumulation in the proximal axons of paclitaxel-treated mice with depletion of mitochondria in distal processes (Smith et al., 2016). These impairments extend to the transport of nuclear mRNA transcripts encoding proteins required for mitochondrial fusion and fission causing mitochondrial vacuolization, decreased ATP production, calcium influx and decreased axon diameter consistent with progression towards cell death (Bobylev et al., 2015). Functionally, these changes translate into decreases in conduction velocity and lengthened conduction latencies in sensory, but not motor, neurons (Jamieson et al., 2007). Disruption of energy production in the distal axon coupled with inhibition of microtubule dynamics halt growth at the distal axon, which regenerates the peripheral processes of terminally-differentiated sensory neurons with turnover of epidermal cells in the skin (Gornstein and Schwarz, 2017). Thus, paclitaxel appears to inhibit the normal regrowth of peripheral termi, which express growth factors (Cheng et al., 2010), into new layers of the epidermis with keratinocyte division (Gornstein and Schwarz, 2017). Therefore, one hallmark of paclitaxel treatment in rodents is decreased intraepidermal nerve fiber
density in the glabrous skin of the paw (Siau et al., 2006; Boyette-Davis et al., 2011; Toma et al., 2017) at time points corresponding to hyper-reflexive behaviors to hindpaw stimulation. Schwann cells surrounding the sciatic nerve indicate disruption of neuronal homeostasis, increasing activating transcription factor-3 (ATF-3) (Peters et al., 2007), and disruptions in neuronal function translate to the cell body of primary sensory afferents located in the DRG.

**Paclitaxel CIPN: Dorsal Root Ganglia Toxicity**

In DRG neurons, paclitaxel triggers both direct neuronal sensitization and inflammation causing secondary neuronal hyperexcitability. While paclitaxel can accumulate in peripheral nerves, paclitaxel concentrations are the highest in the DRG following venous administration (Cavaletti et al., 2000). In the DRG, primary sensory afferents are exposed to high concentrations of paclitaxel while motor neurons in the spinal ventral horn are only exposed to low concentrations of paclitaxel (Xiao et al., 2011). Therefore, the actions of paclitaxel on sensory neurons in the DRG are thought to underlie neuropathy development (Xiao et al., 2011). Paclitaxel-induced structural changes to DRG neurons include nuclear eccentricity and nucleolar enlargement without changes in cell body size or number (Jamieson et al., 2007). Upregulation of activating transcription factor 3 (ATF-3), a marker of neuronal injury, occurs in medium and large neurons of lumbar DRG following paclitaxel treatment (Jimenez-Andrade et al., 2006). Neuronal ATF-3 expression following paclitaxel treatment is highest in the lumbar DRG versus trigeminal or thoracic DRGs, which is consistent with the “stocking-and-glove” distribution of symptoms in patients with paclitaxel CIPN (Dougherty et al., 2004; Jimenez-Andrade et al., 2006). Paclitaxel treatment also causes a hypertrophy of satellite cells, as evidenced by glial fibrillary acidic protein (GFAP) upregulation, and macrophage accumulation in the DRG, both of which occur after neuronal toxicity (Jimenez-Andrade et al., 2006; Peters et al., 2007).
Electrophysiologic analysis of DRG neurons following paclitaxel treatment demonstrates enhanced excitability, including increased spontaneous activity in large- and medium-sized neurons without a change in resting membrane potential (Zhang and Dougherty, 2014). When stratified by neuron type, both myelinated (Aβ and Aδ) and unmyelinated (C) fibers exhibit hyperexcitability due to increased expression of potassium, sodium, calcium and transient receptor potential ion channels (Zhang and Dougherty, 2014). Alterations in sensory processing from paclitaxel treatment are due to both direct receptor signaling and secondary to inflammation.

In addition to microtubule stabilization, paclitaxel has pro-inflammatory properties in cells of the immune system. Treatment of murine macrophages with paclitaxel induces tumor necrosis factor alpha (TNF-α) release similar to that observed by application of bacterial lipopolysaccharide (LPS) (Ding et al., 1990), but independent of the microtubule-stabilizing effects of paclitaxel (Manthey et al., 1993). Similar to LPS, paclitaxel also triggers NF-κB translocation to the nucleus in macrophages (Perera et al., 1996). In lymphocytes, paclitaxel-induced NF-κB expression is due to activation of toll-like receptor 4 (TLR4) (Kawasaki et al., 2000). In macrophages, paclitaxel increases inflammatory microtubule-associated protein kinase (MAPK) activation and NF-κB expression in a TLR4-dependent manner, through molecules-myeloid differentiation primary response gene 88 (MyD88) signaling (Byrd-Leifer et al., 2001).

In the DRG, paclitaxel upregulates TLR4 receptors, increasing signaling through the MyD88 pathway concurrent with the development of mechanical allodynia (Li et al., 2014). Co-treatment of TLR4 receptor antagonist, LPS from Rhodobacter sphaeroides, with paclitaxel prevented allodynia development, suggesting that TLR4 activity is critical for neuropathy (Li et al., 2014). TLR4-dependent signaling from paclitaxel also activates MAPKs in DRG neurons,
including phosphorylation of ERK1/2 and p38 (Li et al., 2015). Phospho-ERK1/2 localized to small diameter CGRP-positive neurons, CGRP/IB4 negative neurons and satellite cells while phospho-p38 was found in small IB4-positive and CGRP+ neurons (Li et al., 2015). Administration of a TLR4 antagonist in conjunction with paclitaxel blocked the phosphorylation of both ERK1/2 and p38 (Li et al., 2015). Inhibition of MAPK signaling, including p38 activity, during paclitaxel treatment prevented allodynia development (Li et al., 2015). Taken together, these studies indicate that paclitaxel exerts direct signaling through TLR4 receptor activation, triggering MyD88 and MAPK signaling cascades, in DRG neurons that is critical for allodynia development.

The impact of paclitaxel in the DRG also leads to the expression of pro-inflammatory cytokines including C-C motif chemokine ligand 2 (CCL2), also termed monocyte chemoattractant protein-1 (MCP-1) (Zhang et al., 2013, 2016a; Makker et al., 2017) in a TLR4-dependent manner (Zhang et al., 2016a). Following paclitaxel treatment, MCP-1 is upregulated in DRG neurons, specifically small- and medium-diameter neurons and, to a lesser extent, large-diameter neurons (Zhang et al., 2013). Additionally, paclitaxel treatment induced expression of the CCR2, the receptor for MCP-1, in myelinated neurons (Zhang et al., 2013). Activation of CCR2 by MCP-1 in these tissues caused a significant increase in intracellular calcium (Zhang et al., 2013), suggesting that MCP-1 release and activation of sensory afferents contributes to paclitaxel neuropathy development and maintenance. Attenuation of MCP-1 expression by anti-MCP-1 antibodies during paclitaxel treatment prevented the development of mechanical allodynia and, after allodynia onset, knockdown of CCR2 reversed allodynia (Zhang et al., 2013). Unexpectedly, blockade of MCP-1 expression during paclitaxel treatment also prevented loss of peripheral nerve fibers in the hindpaw skin (Zhang et al., 2013), suggesting that MCP-1
may mediate intraepidermal nerve fiber loss in addition to other toxic effects of paclitaxel previously discussed.

The actions of MCP-1 in the DRG extend beyond direct neuronal sensitization and include a critical pro-inflammatory role. Treatment with paclitaxel elicits macrophage accumulation in the DRG (Peters et al., 2007) in a time-dependent manner that corresponds with the development of mechanical allodynia (Zhang et al., 2016a). Administration of anti-MCP-1 antibodies during paclitaxel treatment blocked macrophage recruitment to the DRG and allodynia development, suggesting that MCP-1 expression exerts a chemotactic effect (Zhang et al., 2016a). A TLR4 receptor antagonist attenuated these effects, suggesting that MCP-1 release in the DRG may also be a consequence of TLR4 receptor activation. Accumulation of macrophages in the DRG causes an increase in TNF-α release (Zhang et al., 2016a).

**Paclitaxel CIPN: Spinal Dorsal Horn Toxicity**

In addition to neuropathy development in the peripheral nervous system, paclitaxel causes significant changes in the spinal dorsal horn, containing the termini of primary afferents, cell bodies of secondary afferents, excitatory and inhibitory interneurons modulating dorsal horn signalling, and glial cells (D’Mello and Dickenson, 2008). In the nervous system, paclitaxel concentrations are highest in the DRG after administration while low concentrations are found in the spinal cord and may be from peripheral afferent termini (Cavaletti et al., 2000). The lowest concentrations of paclitaxel are found in the brain (Cavaletti et al., 2000) as paclitaxel is restricted from the brain due to efflux by the multidrug resistance protein p-glycoprotein (Fellner et al., 2002). In rat brain capillaries, paclitaxel diffuses into vascular endothelial cells, but is excreted into the vessel lumen in an energy-dependent manner (Fellner et al., 2002). While paclitaxel levels could not be quantified in the brains of mice treated with intravenous paclitaxel...
(8 mg/kg), inhibition of p-glycoprotein with valspodar caused a significant increase in brain paclitaxel levels for at least 24 hours after treatment (Fellner et al., 2002). This suggests that the vascular endothelium limits paclitaxel entry into the brain by expressing a p-glycoprotein transporter (Fellner et al., 2002).

In the spinal cord, paclitaxel treatment causes enhanced neuronal excitability. Long-term potentiation in C-fibers is enhanced following paclitaxel treatment (Zhu et al., 2015). Wide dynamic range neurons, which receive input from multiple sensory fiber types (D’Mello and Dickenson, 2008), exhibit both stimulus-independent spontaneous activity and enhanced afterdischarges to mechanical stimulation following paclitaxel treatment (Cata et al., 2006).

Inflammatory changes in the spinal cord following paclitaxel treatment include astrocyte activation and, depending on the study design, microglial activation, at time points corresponding with behavioral hypersensitivity. Paclitaxel treatment induces a robust increase in GFAP expression in the superficial dorsal horn (Zhang et al., 2012), with decreased expression of the glial glutamate transporters GLAST and GLT-1, disrupting glutamate homeostasis (Weng et al., 2005; Zhang et al., 2012). An increase in GFAP expression in the dorsal horn following paclitaxel treatment is widely (Peters et al., 2007; Zhang et al., 2012; Rahn et al., 2014; Makker et al., 2017) reported between four hours and 28 days after the start of paclitaxel treatment. However, one group did not observe GFAP expression reported three days after the start of paclitaxel treatment (Pevida et al., 2013). Compared to astrocyte activation, the contribution of microglia is less clear. While some groups report increased CD11b (Peters et al., 2007) between 6-40 days after the start of paclitaxel treatment or Iba-1 (Pevida et al., 2013) immunoreactivity in the spinal dorsal horn three days after the start of paclitaxel treatment, others report no change in CD11b/OX42 (Zhang et al., 2012; Rahn et al., 2014), Iba-1, or phospho-p38 expression (Zhang
et al., 2012) within 28 days after the start of paclitaxel treatment. Additionally, paclitaxel treatment does not elicit pro-inflammatory cytokine (TNF-α, IL-1β or IL-6) expression production in the spinal dorsal horn (Zhang et al., 2012; Deng et al., 2015b) four hours, seven days or 15 days after the start of paclitaxel treatment. While phosphorylation of p38 is not microglia-specific, it is of note that the same group reports an upregulation in phospho-p38 expression in DRG neurons (Li et al., 2015), but not in the spinal dorsal horn (Zhang et al., 2012; Li et al., 2015). While TLR4 is upregulated in the dorsal horn one through 21 days after the start of paclitaxel treatment, it is limited to astrocytes (Li et al., 2014). In the DRG, the upregulation of TLR4 occurs in neurons (Li et al., 2014) for up to one week after starting paclitaxel treatment where the phosphorylation of p38 is observed one and two weeks after starting paclitaxel treatment (Li et al., 2015). One marker of inflammation in the spinal cord that is consistent with the DRG expression is MCP-1 (Zhang et al., 2013).

In the spinal cord, MCP-1 expression is induced in astrocytes, but not neurons which express MCP-1 in the DRG (Zhang et al., 2013). As the CCR2 receptor is not upregulated in the spinal cord (Zhang et al., 2013), whether or not spinal MCP-1 contributes to the development of central sensitization is less clear. It is also possible that MCP-1 expression may be due to neuronal or microglia expression as one group reports an increase in spinal MCP-1 in paclitaxel-treated animals without astrocyte activation (Pevida et al., 2013). While intrathecal administration of antibodies targeting MCP-1 reverses paclitaxel-induced mechanical allodynia, these effects may be due to MCP-1 in the DRG (Zhang et al., 2016a).

The Endogenous Cannabinoid System: Potential Targets to Treat CIPN

Administration of paclitaxel or cisplatin to mice produces changes in the endogenous cannabinoid system that differ between the two chemotherapeutic agents. Following paclitaxel
treatment, CB₁ receptor, the CB₂ receptor, FAAH and MAGL mRNA levels were unchanged in the lumbar spinal cord 15 days after starting paclitaxel (Deng et al., 2015b). However, quantification of the endocannabinoids and related lipids in the lumbar spinal cord was not conducted after paclitaxel treatment. Following cisplatin administration, MAGL, CB₁ receptor, or CB₂ receptor mRNA expression is not altered compared to control mice; however, FAAH mRNA expression is elevated in the lumbar spinal cord 16 days after the start of cisplatin treatment (Guindon et al., 2013). Quantification of endogenous cannabinoids in the lumbar spinal cord following cisplatin treatment demonstrates increases in both AEA and 2-AG levels (Guindon et al., 2013), suggesting that changes in endocannabinoid levels due to cisplatin administration may also involve other biosynthetic or degradative enzymes. In the DRG, cisplatin did not alter FAAH, MAGL, CB₁ receptor or CB₂ receptor mRNA expression (Guindon et al., 2013).

In addition to insights into neuropathy development and maintenance, animal models of CIPN serve as screening assays for therapeutic development. Modulation of the endogenous cannabinoid system in animal models of paclitaxel, cisplatin and vincristine CIPN can identify targets for further therapeutic development. These studies are also useful for understanding the mechanisms underlying CIPN development and maintenance in rodent models. To date, studies have examined the endogenous cannabinoid system in the reversal of established mechanical, cold and thermal hyperreactivity in rodent models of CIPN. These studies are outlined in Table 1. Prevention of allodynia development with these agents will be discussed in the next section.
Table 1. Review of literature demonstrating reversal of established allodynia or hyperalgesia in rodent models of CIPN by drugs targeting the endogenous cannabinoid system

<table>
<thead>
<tr>
<th>Target Drug</th>
<th>Species</th>
<th>Mechanical</th>
<th>Heat</th>
<th>Cold</th>
<th>Receptor Involvement (Yes/No)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel CIPN</td>
<td>CB₁ &amp; CB₂</td>
<td>CP55,940 Mouse</td>
<td>+</td>
<td>+</td>
<td>Y Y</td>
<td>(Deng et al., 2015a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WIN Rat</td>
<td>+</td>
<td>+</td>
<td>Y</td>
<td>(Pascual et al., 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>THC Mouse</td>
<td>+</td>
<td>+</td>
<td></td>
<td>(Deng et al., 2015b; King et al., 2017)</td>
</tr>
<tr>
<td></td>
<td>CB₂</td>
<td>AM1241 Rat</td>
<td>+</td>
<td></td>
<td>N Y</td>
<td>(Rahn et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AM1710 Mouse, Rat</td>
<td>+</td>
<td>+</td>
<td>N Y</td>
<td>(Deng et al., 2012, 2015b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AM1714 Rat</td>
<td>+</td>
<td></td>
<td>N Y</td>
<td>(Rahn et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MDA7 Rat</td>
<td>+</td>
<td></td>
<td></td>
<td>(Naguib et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MDA19 Mouse, Rat</td>
<td>+</td>
<td></td>
<td>Y</td>
<td>(Xu et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>MAGL</td>
<td>JZL184 Mouse</td>
<td>+</td>
<td>+</td>
<td></td>
<td>(Slivicki et al., 2017)</td>
</tr>
<tr>
<td></td>
<td>FAAH</td>
<td>URB597 Mouse</td>
<td>+</td>
<td>+</td>
<td></td>
<td>(Slivicki et al., 2017)</td>
</tr>
<tr>
<td></td>
<td>DAGLβ</td>
<td>KT-109 Mouse</td>
<td>+</td>
<td></td>
<td></td>
<td>(Wilkerson et al., 2016a)</td>
</tr>
<tr>
<td>Cisplatin CIPN</td>
<td>CB₁ &amp; CB₂</td>
<td>AEA Mouse</td>
<td>+</td>
<td></td>
<td>Y N</td>
<td>(Khasabova et al., 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>THC Mouse</td>
<td>+</td>
<td></td>
<td></td>
<td>(Harris et al., 2016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WIN Rat</td>
<td>+</td>
<td></td>
<td>Y Y/N</td>
<td>(Vera et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>CB₂</td>
<td>AM1710 Rat</td>
<td>+</td>
<td>+</td>
<td>N Y</td>
<td>(Deng et al., 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JWH133 Rat</td>
<td>+</td>
<td></td>
<td>Y</td>
<td>(Vera et al., 2013)</td>
</tr>
</tbody>
</table>
FAAH  URB597  Mouse, Rat  +  +  +  Y  Y/N  (Khasabova et al., 2012; Guindon et al., 2013)
                               URB937  Rat  +  +  Y  Y  (Guindon et al., 2013)
                               MAGL  JZL184  Mouse, Rat  +  +  Y  Y/N  (Guindon et al., 2013; Khasabova et al., 2014)

Vincristine CIPN
   CB1 & CB2  THC  Mouse  +  (King et al., 2017)
             WIN  Rat  +  Y  Y  (Rahn et al., 2007)
             CB2  AM1241  Rat  +  N  Y  (Rahn et al., 2007)
                               FAAH  ST4070  Rat  +  (Caprioli et al., 2012)

Positive anti-hypersensitivity results for mechanical, thermal and cold stimuli are denoted with a “+”. In general, the mechanical stimuli refer to von Frey filament, pressure (Randal Stiletto) or similar testing. Thermal stimuli generally included focused beams of light, hotplate plantar withdrawal or tail flick, depending on the type of neuropathy. Cold stimuli referred to positive results using plantar acetone, cold plate or similar testing. Cannabinoid (CB1 or CB2) receptor involvement includes results from mice lacking the gene of interest or experiments using pharmacologic antagonists. Full or partial blockade of analgesic effects is denoted with a “Y” (yes). Negative results are reported with an “N” (no). Delta-9-tetrahydrocannabinol; THC. WIN55,212.; WIN. Adapted from (Donvito et al., 2017).

In rodents, direct agonists of the CB1 and CB2 receptors (mixed agonists) reverse paclitaxel-induced changes in mechanical, thermal and cold sensitivity. Administration of Δ9-tetrahydrocannabinol (THC) reduced mechanical allodynia in mice (Deng et al., 2015b). The mixed agonist WIN55,212-2 reversed both mechanical allodynia and heat hyperalgesia in a rat model of paclitaxel CIPN in a CB1-dependent manner (Pascual et al., 2005). However, the contribution of CB2 receptors to this effect was not assessed (Pascual et al., 2005). Similarly, the mixed agonist CP55,940 reversed mechanical and cold allodynia in mice (Deng et al., 2015a). Interestingly, this effect was differentially regulated by the cannabinoid receptors. At a low dose (0.3 mg/kg, daily), CP55,940 reversed mechanical and cold allodynia in control and CB2 (-/-) mice, but not in CB1 (-/-) mice. Administration of a high dose (10 mg/kg, daily) produced anti-
allodynic effects in CB1 (-/-) mice but led to the development of catalepsy in (+/+)
mice. Therefore, the CB1 receptor mediates the anti-allodynic effects of the low-dose CP55,940 with
additional anti-allodynic effects of at the higher dose CP55,940 that could be CB2-dependent
however direct assessment of this dose in CB2 (-/-) mice would be hampered by catalepsy
development.

The use of selective CB2 receptor agonists demonstrates a role for the CB2 receptor in the
reversal of paclitaxel allodynia. The direct agonists AM1241 (Rahn et al., 2008), AM1710 (Deng
et al., 2012, 2015b) and AM1714 (Rahn et al., 2008) reversed paclitaxel-induced mechanical
and/or cold (only AM1710 tested) allodynia in rodent models in a CB2-dependent manner.
Administration of AM1710 also suppressed paclitaxel-induced increases in MCP-1 in the lumbar
spinal cord, suggesting a possible mechanism of neuropathy reversal (Deng et al., 2015b).

In addition to direct receptor agonists, inhibitors of the enzymes MAGL and FAAH,
degrading 2-AG and AEA, respectively, reversed mouse paclitaxel-induced mechanical and cold
allodynia (Slivicki et al., 2017). Both JZL184 (MAGL) and URB597 (FAAH) produced
synergistic anti-allodynic effects with the CB1 positive allosteric modulator, GAT211 (Slivicki et
al., 2017). Paradoxically, inhibition of DAGLβ, which decreases 2-AG levels in macrophages
and microglia (Hsu et al., 2012), also exhibit anti-allodynic effects in mouse paclitaxel CIPN
(Wilkerson et al., 2016a). This could be due to decreased arachidonic acid and prostaglandin
levels in the CNS secondary to decreased 2-AG levels (Nomura et al., 2011b). However, because
cyclooxygenase inhibition does not reverse mechanical allodynia from paclitaxel (Ito et al.,
2012), these effects may be due to decreased inflammatory mediators separate from those
produced by the cyclooxygenase pathway.
As with paclitaxel CIPN, administration of agents modulating the endogenous cannabinoid system also attenuates allodynia in models of cisplatin CIPN. In cisplatin-treated rodents, the mixed (CB₁ and CB₂ receptor) agonist AEA reversed mechanical allodynia in a CB₁-dependent manner (Khasabova et al., 2012). THC also reversed mechanical allodynia in cisplatin-treated mice (Harris et al., 2016). The mixed agonist WIN55,212-2 attenuated cisplatin-induced mechanical allodynia in CB₁ and CB₂-dependent manners with intraperitoneal administration, but only reversed allodynia in a CB₁-dependent manner with intraplantar administration (Vera et al., 2013).

The use of selective CB₂ receptor-selective agonists, FAAH inhibitors and MAGL inhibitors also demonstrates a role for the CB₂ receptor in the reversal of cisplatin CIPN. Both the CB₂ receptor direct agonists AM1710 (Deng et al., 2012) and JWH133 (Vera et al., 2013) reversed mechanical allodynia from cisplatin treatment in CB₂-dependent manners. AM1710 also attenuated cisplatin cold allodynia in a CB₂-dependent manner (Deng et al., 2012). The FAAH inhibitors URB597 (Khasabova et al., 2012; Guindon et al., 2013) and URB937 (Guindon et al., 2013) reversed cisplatin-induced mechanical allodynia in CB₁- and CB₂-dependent manners. The CB₂ receptor contribution to mechanical allodynia reversal was not observed by (Khasabova et al., 2012), but may be due to intraplantar administration—causing local elevations in AEA—versus intraperitoneal administration (Guindon et al., 2013) causing systemic elevations in AEA that would include the CNS. Procedural differences may also contribute to differences in the mechanism(s) of alldynia development in the spinal cord and DRG. In the study by (Khasabova et al., 2012), cisplatin was administered daily at 1 mg/kg for seven days in C3H/HeN mice versus 3 mg/kg once a week for three weeks in Sprague-Dawley rats (Guindon et al., 2013). However, it is unknown if these administrations procedures alter
cisplatin neuropathy development. FAAH inhibition also reversed heat hyperalgesia (Khasabova et al., 2012) and cold allodynia (Guindon et al., 2013) from cisplatin treatment. These same groups also tested the MAGL inhibitor JZL184, which has higher potency for mouse MAGL versus rat MAGL (Long et al., 2009b). MAGL inhibition reversed both mechanical (Guindon et al., 2013; Khasabova et al., 2014) and cold (Guindon et al., 2013) allodynia from cisplatin treatment. Mechanical allodynia reversal was CB1-dependent with both intraperitoneal (Guindon et al., 2013) and intraplantar administration of JZL184 (Khasabova et al., 2014). Mechanical allodynia reversal was also CB2-dependent, but only with intraperitoneal (Guindon et al., 2013)—causing systemic increases in 2-AG including the CNS—versus intraplantar (Khasabova et al., 2014) administration.

As with cisplatin neuropathy, drugs targeting various components of the endogenous cannabinoid system also reverse vincristine-induced mechanical allodynia. Mixed (CB1 and CB2 receptor) agonists reverse vincristine-induced mechanical allodynia. Both THC (King et al., 2017) and WIN55,212-2 reversed mechanical allodynia, the latter in a CB1- and CB2-dependent manner (Rahn et al., 2007). The direct CB2 agonist, AM1241, also reversed vincristine mechanical allodynia in a CB2-dependent manner (Rahn et al., 2007). Lastly, the FAAH inhibitor ST4070 reversed mechanical allodynia, though the contribution of the cannabinoid receptors to this effect was not assessed (Caprioli et al., 2012).

**The Endogenous Cannabinoid System: Potential Targets to Prevent CIPN**

In addition to reversing mechanical allodynia from paclitaxel administration, modulation of the endogenous cannabinoid system can prevent the development of allodynia prior to onset, summarized in table 2 with drug administration protocols listed with results. In rats, co-treatment of the dual CB1/CB2 agonist WIN55,212-2 with paclitaxel prevented the development of
mechanical allodynia (Burgos et al., 2012), heat hyperalgesia (Burgos et al., 2012) and cold allodynia (Rahn et al., 2014) following cessation of paclitaxel treatment. Mechanical allodynia and heat hyperalgesia were prevented for the entirety of one study, 28 days after cessation of 1 mg/kg paclitaxel, given on alternate days (Burgos et al., 2012). Conversely, prevention of mechanical allodynia and cold allodynia only lasted for about one week following cessation of 2 mg/kg paclitaxel, given on alternate days (Rahn et al., 2014). While the prevention of cold allodynia with WIN55,212-2 is CB₁ and CB₂-independent, prevention of mechanical allodynia development is CB₁-mediated (Rahn et al., 2014). Co-treatment with WIN55,212-2 also prevented inflammation in the spinal cord, including paclitaxel-induced astrocyte activation (GFAP expression), microglia activation (CD11b expression), and pro-inflammatory cytokine (IL-1β, IL-6, TNFα) expression (Burgos et al., 2012) following treatment with 1 mg/kg paclitaxel, given on alternate days. However, when this was increased to 2 mg/kg paclitaxel, microglia activation (CD11b expression) was not increased by paclitaxel alone and astrocyte activation (GFAP expression) was not prevented with WIN55,212-2 co-treatment (Rahn et al., 2014).

While WIN55,212-2 prevented mechanical allodynia in a CB₁-dependent manner (Rahn et al., 2014), the CB₂ agonists MDA7 (Naguib et al., 2012) and AM1710 (Rahn et al., 2014) prevented allodynia development from paclitaxel treatment. Co-administration of the direct CB₂ agonists MDA7 or AM1710 with paclitaxel prevented mechanical and cold (AM1710 only) allodynia during and up to two weeks after paclitaxel treatment (Naguib et al., 2012; Rahn et al., 2014). Prevention of mechanical (Naguib et al., 2012; Rahn et al., 2014) and cold (Rahn et al., 2014) allodynia with MDA7 or AM1710 was blocked by co-administration of a CB₂ antagonist. Mechanical allodynia prevention with MDA7 was also not observed in CB₂ (-/-) mice compared
to (+/+) controls (Naguib et al., 2012). In the spinal dorsal horn, MDA7 prevented astrocyte activation (GFAP expression) and microglia activation (CD11b expression) from paclitaxel administration 14 days after the last paclitaxel treatment (Naguib et al., 2012) in addition to decreasing pro-inflammatory cytokine (IL-1β, IL-6 and TNF-α) levels (Xu et al., 2014). One group also reports that paclitaxel treatment increased CB2 receptor expression in reactive astrocytes, an effect prevented by co-treatment with MDA7 (Naguib et al., 2012). However, increased CB1 or CB2 receptor expression in the spinal dorsal horn is not observed by all groups (Rahn et al., 2014). This may also be due to differences in paclitaxel dose with the former group using 1 mg/kg on four alternate days versus 2 mg/kg for the latter. Lastly, cannabidiol, which is also found in cannabis (Mechoulam et al., 1970), prevents the development of paclitaxel-induced mechanical allodynia up to 52 days after cannabidiol cessation and cold allodynia up to 39 days after cannabidiol cessation (Ward et al., 2011). Prevention of allodynia with cannabidiol is cannabinoid receptor-independent, as evidenced by co-treatment with CB1 and CB2 receptor antagonists, but is serotonin 1A (5-HT1A) receptor-mediated (Ward et al., 2014).

While inhibitors of endocannabinoid hydrolysis have not been tested in the prevention of mechanical allodynia from paclitaxel treatment, the FAAH inhibitor URB597 prevented cisplatin-induced mechanical allodynia and heat hyperalgesia in a CB1-dependent manner with seven days of co-administration (Khasabova et al., 2012). Treatment with URB597 also attenuated cisplatin-induced ATF-3 expression in the DRG and prevented a decrease in conduction velocity in neurons isolated from the sciatic nerve (Khasabova et al., 2012). However, URB597 does inhibit other serine hydrolases in addition to FAAH (Ahn et al., 2009). Similarly, the MAGL inhibitor JZL184 prevented the development of mechanical allodynia from cisplatin treatment in a CB1-dependent manner with seven days of co-administration (Khasabova
et al., 2014). However, for both URB597 and JZL184 in cisplatin neuropathy, these studies did not examine prevention of allodynia after cessation of cisplatin treatment. The direct CB₁/CB₂ agonist WIN55,212-2 also prevented cisplatin-induced mechanical allodynia up to four days after the last cisplatin treatment (Vera et al., 2007). For vincristine CIPN, only THC has been evaluated, with partial prevention of alldynia up to 22 days after co-administration compared to control (King et al., 2017).
Table 2. Review of literature demonstrating prevention of allodynia and hyperalgesia behaviors in rodent models of CIPN by drugs targeting the endogenous cannabinoid system

<table>
<thead>
<tr>
<th>Target</th>
<th>Drug</th>
<th>Species</th>
<th>Drug Administration Protocol and Duration of Prevention</th>
<th>Receptor Involvement (Yes/No)</th>
<th>Reference(s)</th>
</tr>
</thead>
</table>
| Paclitaxel CIPN | WIN   | Rat     | **WIN**: 1 mg/kg daily for 14 days, starting with paclitaxel  
**Paclitaxel**: 1 mg/kg, four alternate days  
**MA and TH** prevented for 28 days after paclitaxel cessation  
**CD11b and GFAP** expression in the spinal dorsal horn prevented on day 29  
**IL-1β, IL-6 and TNF-α** expression in the spinal cord prevented four days after the start of the study | CB₁ (Y) CB₂ (N) | (Burgos et al., 2012) |
| WIN          | Rat   | WIN     | **WIN**: 0.1, 0.5 and 1 mg/kg, s.c. daily for 26 days via osmotic mini-pump. Six days of pre-treatment prior to paclitaxel  
**Paclitaxel**: 2 mg/kg, i.p., four alternate days  
**MA** prevented by 0.5 mg/kg WIN for 14 days after paclitaxel cessation. 0.1 and 1 mg/kg WIN only partially prevented MA. MA prevented up to 11 days after WIN withdrawal.  
**CA** prevented by 0.1 and 0.5 mg/kg for 14 days after paclitaxel cessation. 1 mg/kg WIN only partially prevented CA. CA prevented up to 12-18 days after WIN withdrawal. | CB₁ (Y) CB₂ (N) | (Rahn et al., 2014) |
| THC          | Mouse | THC     | **THC**: 0.625-20 mg/kg, i.p. 15 minutes prior to paclitaxel  
**Paclitaxel**: 8 mg/kg, i.p., four alternate days  
**MA** prevented on day 1 post-paclitaxel by 2.5, 10 and 20 mg/kg THC and on day 6 by 10 and 20 mg/kg THC. No prevention on day 13 | CB₁ (N) CB₂ (N) | (King et al., 2017) |
| CB2 AM1710 Rat | AM1710: 0.032, 0.32 and 3.2 mg/kg, s.c. daily for 28 days via osmotic minipump. Six days of pre-treatment prior to paclitaxel | Paclitaxel: 2 mg/kg, i.p., four alternate days | MA prevented by 0.032 and 3.2 mg/kg for 14 days after paclitaxel cessation. MA prevented up to 17 days after WIN withdrawal. CA prevented by 3.2 mg/kg for 14 days after paclitaxel cessation. CA prevented for up to 18 days after AM1710 withdrawal. | N Y (Rahn et al., 2014) |
| MDA7 Rat, Mouse | MDA7: 15 mg/kg, i.p. daily for 4 or 14 days, starting with paclitaxel | Paclitaxel: 1 mg/kg, i.p., four consecutive days | MA prevented by 14 days, but not 4 days, of co-treatment up to 14 days after MDA7 cessation. CD11b and GFAP expression in the spinal dorsal horn prevented on day 28 TNFα, IL-1β and IL-6 expression in the spinal dorsal horn prevented one day after the last MDA7 treatment | Y (Naguib et al., 2012; Xu et al., 2014) |
| 5-HT1A Cannabidiol Mouse | CBD: 5 or 10 mg/kg, i.p. daily for 14 days, starting with paclitaxel | Paclitaxel: 8 mg/kg, i.p., four alternate days | MA prevented beginning one day after the last paclitaxel treatment and up to 52 days after CBD cessation CA prevented beginning two days after the last paclitaxel treatment up to 39 days after CBD cessation. CBD: 2 or 5 mg/kg, i.p. 15 minutes prior to paclitaxel Paclitaxel: 8 mg/kg, i.p., four alternate days MA prevented beginning one day after the last paclitaxel treatment and up to 10 weeks after co-treatment. Prevention is CB1- and CB2-independent but appears to be 5-HT1A receptor-mediated. CBD: 0.625-20 mg/kg, i.p. 15 minutes prior to paclitaxel Paclitaxel: 8 mg/kg, i.p., four alternate days MA prevented on days 1 and 6 post-paclitaxel by 1 and 20 mg/kg CBD, but not on day 13 | N N (Ward et al., 2011) (Ward et al., 2014) (King et al., 2017) |
### Cisplatin CIPN

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Compound</th>
<th>Species</th>
<th>Dose</th>
<th>Route</th>
<th>Duration</th>
<th>Timing</th>
<th>Treatment Details</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB&lt;sub&gt;1&lt;/sub&gt; &amp; CB&lt;sub&gt;2&lt;/sub&gt;</td>
<td>WIN</td>
<td>Rat</td>
<td>WIN: 1 or 2 mg/kg, i.p. once a week for five weeks, 30 minutes prior to cisplatin</td>
<td>Cisplatin: 1 or 2 mg/kg, i.p. once a week for five weeks</td>
<td>MA prevented by 1 and 2 mg/kg WIN four days after the last treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAAH</td>
<td>URB597</td>
<td>Mouse</td>
<td>URB597: 0.3 mg/kg daily, i.p. for seven days</td>
<td>Cisplatin: 1 mg/kg daily, i.p. for seven days</td>
<td>MA and TH prevented relative to cisplatin control for seven days. No data after URB597 or cisplatin cessation.</td>
<td>ATF-3 expression in the DRG prevented on day seven</td>
<td>Conduction velocity</td>
<td>Sciatic nerve neurons, decreased with cisplatin treatment, is spared with URB597 co-treatment</td>
</tr>
<tr>
<td>MAGL</td>
<td>JZL184</td>
<td>Mouse</td>
<td>JZL184: 10 µg, s.c. dorsal hind paw, daily for seven days</td>
<td>Cisplatin: 1 mg/kg daily, i.p. for seven days</td>
<td>MA prevented relative to cisplatin control for seven days. No data after JZL184 or cisplatin cessation.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Vincristine CIPN

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Compound</th>
<th>Species</th>
<th>Dose</th>
<th>Route</th>
<th>Duration</th>
<th>Timing</th>
<th>Treatment Details</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB&lt;sub&gt;1&lt;/sub&gt; &amp; CB&lt;sub&gt;2&lt;/sub&gt;</td>
<td>THC</td>
<td>Mouse</td>
<td>THC: 10 mg/kg, i.p. 15 minutes prior to vincristine</td>
<td>Vincristine: 0.1 mg/kg, i.p., daily for 7 days</td>
<td>MA only partially prevented on days 5, 10, 15 and 22 after start of treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mechanical allodynia (MA) refers to von Frey filament or similar testing. Cold allodynia (CA) referred to positive results using plantar acetone or similar testing. Thermal (heat) hyperalgesia (TH) included paw withdrawal to focused beams of light or hotplate stimuli. Days after drug treatment are as reported or estimated from figures and methods. Cannabinoid (CB<sub>1</sub> or CB<sub>2</sub>) receptors involvement includes results from mice lacking the gene of interest or experiments using pharmacologic antagonists. Full or partial blockade of analgesic effects is denoted with a “Y” (yes). Negative results are reported with an “N” (no). 5-HT<sub>1A</sub>; serotonin 1A receptor. Cannabidiol; CBD. Delta-9-tetrahydrocannabinol; THC. WIN55,212.; WIN.
**Hypothesis**

MAGL inhibition will reverse the expression of mouse paclitaxel-induced mechanical allodynia and markers of inflammation after onset in addition to preventing allodynia development through cannabinoid receptor mechanisms of action.

![Diagram](image)

**Figure 3. Diagram of hypothesis.** Monoacylglycerol lipase (MAGL) inhibition will alleviate mouse paclitaxel-induced mechanical allodynia and markers of inflammation through cannabinoid receptor (CB type 1 or 2) mechanisms of action.

**Mouse Model of Paclitaxel CIPN**

In this work, we examine the effectiveness of MAGL inhibitors to reverse and prevent mouse paclitaxel-induced mechanical allodynia. We chose an established mouse model of paclitaxel neuropathy (Toma et al., 2017) previously employed by our laboratory (Donvito et al., 2016) and elsewhere (King et al., 2017) to study modulation of the endocannabinoid system. Injection of 8 mg/kg (i.p) of paclitaxel on four alternate days produced bilateral mechanical and cold allodynia to hind paw stimulation in C57BL/6J mice. Mechanical allodynia, assessed using von Frey filaments, was stable over the course of eleven weeks post-paclitaxel, while cold allodynia, assessed using acetone, was only
observed for up to three weeks post-treatment (Toma et al., 2017). This model also produced a preference for the dark chamber of the light/dark box and immobility in the forced swim test, suggesting the development of an affective state from paclitaxel administration (Toma et al., 2017). Given the reliability and duration of mechanical allodynia from this model, as well as extensive studies using MAGL inhibitors with this dependent measure (Schlosburg et al., 2010; Ghosh et al., 2013; Ignatowska-Jankowska et al., 2015; Wilkerson et al., 2016b), we chose mechanical allodynia for behavioral testing. We also expanded previous studies utilizing the CPP paradigm in cisplatin CIPN (Park et al., 2013; Krukowski et al., 2017) by evaluating a candidate analgesic in our paclitaxel model of CIPN.

**Selection of MAGL inhibitors**

To examine MAGL inhibition in reversal and prevention of paclitaxel CIPN, we chose the selective and potent MAGL inhibitors JZL184 and MJN110. Administration of either JZL184 (Long et al., 2009a) or MJN110 (Niphakis et al., 2013) produces a dose-dependent increase in 2-AG and a decrease in arachidonic acid in the central nervous system of mice. MJN110 is more potent, with an IC\(_{50}\) value of 2.1 nM for 2-AG hydrolysis (Niphakis et al., 2013), versus JZL184, with an IC\(_{50}\) value of 8 nM for MAGL (Long et al., 2009b). JZL184 also inhibits FAAH, IC\(_{50}\) = 4 µM, which does not elevate AEA levels with acute administration (Long et al., 2009a), but increases AEA levels with repeated administration (Kinsey et al., 2013). MJN110 does not inhibit FAAH, IC\(_{50}\) >10 µM for AEA hydrolysis, but inhibits ABHD6 (Niphakis et al., 2013), an enzyme that hydrolyzes less than 5% of 2-AG (Blankman et al., 2007). We control for this off-target effect of MJN110 using the selective and potent ABHD6 inhibitor KT195 (Hsu et al., 2012; Wilkerson et al., 2016a).

**Chapter 2 Overview: Monoacylglycerol lipase inhibitors reverse paclitaxel-induced nociceptive behavior and proinflammatory markers in a mouse model of chemotherapy-induced neuropathy**

In Chapter 2, we test the hypothesis that MAGL inhibition will reverse mouse paclitaxel-induced mechanical allodynia through cannabinoid receptor mechanisms of action. As the MAGL inhibitor JZL184 reverses paclitaxel-induced allodynia (Slivicki et al., 2017), we tested whether MJN110 will
also reverse paclitaxel allodynia (Figure 3). Because both MJN110 and JZL184 require CB\(_1\) and CB\(_2\) receptors to mediate their anti-allodynic effects in the CCI model of neuropathic pain (Ignatowska-Jankowska et al., 2015), we tested whether their anti-allodynic effects in paclitaxel CIPN also require cannabinoid receptors using CB\(_1\) and CB\(_2\) \((-/-)\) and matched \((+/+)\) mice in addition to pharmacologic antagonism of CB\(_1\) and CB\(_2\) receptors. As paclitaxel CIPN can last for years after chemotherapy (Tanabe et al., 2013), we predict that potential therapy with a MAGL inhibitor would require repeated treatment. Because prolonged inhibition of MAGL is known to produce tolerance to anti-allodynic effects in models of CCI neuropathy due to decreased CB\(_1\) expression and function (Schlosburg et al., 2010; Kinsey et al., 2013), we predicted that repeated administration of high-dose JZL184 will produce tolerance to its anti-allodynic effects in paclitaxel CIPN. We also tested a strategy to circumvent tolerance development, previously described, using repeated administration of a low-dose of JZL184. This administration protocol reversed CCI-induced allodynia without tolerance development, while sparing CB\(_1\) expression and function (Kinsey et al., 2013). Because the levels of endogenous cannabinoids in the spinal cord have not been quantified following paclitaxel treatment, we examined whether paclitaxel increases AEA and 2-AG levels as observed with cisplatin neuropathy (Guindon et al., 2013) and the impact of JZL184 administration. As a direct CB\(_2\) receptor agonist attenuates increased MCP-1 expression in the lumbar spinal cord of paclitaxel-treated mice (Deng et al., 2015b), we predicted that MAGL inhibition will reverse both spinal and DRG MCP-1 (Zhang et al., 2013; Makker et al., 2017) and DRG phospho-p38 (Li et al., 2015) associated with neuropathy. We also predict that paclitaxel-treated mice, but not control mice, will acquire a preference for a chamber paired with a MAGL inhibitor in the CPP paradigm. Lastly, we test the hypothesis that MAGL inhibitors will not interfere with, but could potentially enhance, the anti-proliferative and anti-apoptotic effects of paclitaxel in cell lines of non-small cell lung cancer.
Chapter 3 Overview: Monoacylglycerol lipase inhibitors prevent the development of paclitaxel-induced mechanical allodynia in mice

In Chapter 3, we test the hypothesis that MAGL inhibition during paclitaxel treatment will prevent the development of mechanical allodynia. This hypothesis is based on previous work examining direct acting cannabinoid receptor agonists as well as inhibitors of MAGL. Specifically, the mixed cannabinoid receptor agonist WIN55,212-2 (Rahn et al., 2014) and the direct CB\textsubscript{2} receptor agonists AM1710 (Rahn et al., 2014) and MDA7 (Naguib et al., 2012) prevent paclitaxel mechanical allodynia prior to development. Additionally, the MAGL inhibitor JZL184 prevents mechanical allodynia development from cisplatin administration (Khasabova et al., 2014). Thus, we tested whether the MAGL inhibitors JZL184 and MJN110, given concurrently with paclitaxel, will prevent the development of mechanical allodynia. Because tolerance can develop to repeated administration of a high dose of JZL184 (Schlosburg et al., 2010), we also employ a low-dose treatment regimen (Kinsey et al., 2013) concurrent with paclitaxel. We also test whether the CB\textsubscript{1} receptor is required for prevention of allodynia with high-dose JZL184 treatment through the use of CB\textsubscript{1} (−/−) and (+/+ ) mice.
Chapter 2: Monoacylglycerol lipase inhibitors reverse paclitaxel-induced nociceptive behavior and proinflammatory markers in a mouse model of chemotherapy-induced neuropathy

Zachary A Curry, Jenny L Wilkerson, Deniz Bagdas, S Lauren Kyte, Nipa Patel, Giulia Donvito, Mohammed A Mustafa, Justin L Poklis, Micah J Niphakis, Ku-Lung Hsu, Benjamin F Cravatt, David A Gewirtz, M Imad Damaj, Aron H Lichtman

Affiliations:
Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA, USA (ZAC, JLW, DB, MAM, SLK, NP, GD, JLP, DAG, MID, AHL)

The Skaggs Institute for Chemical Biology and Department of Chemical Physiology, The Scripps Research Institute, La Jolla, CA, USA (MJN, BFC)

Department of Chemistry, University of Virginia, Charlottesville, VA, USA (KLH)

Adapted from manuscript accepted by the Journal of Pharmacology and Experimental Therapeutics. March 14, 2018; jpet.117.245704; DOI: https://doi.org/10.1124/jpet.117.245704. Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. All rights reserved.
Abstract

Although paclitaxel effectively treats various cancers, its debilitating peripheral neuropathic pain side effects often persist long after treatment has ended. Therefore, a compelling need exists for the identification of novel pharmacologic strategies to mitigate this condition. As inhibitors of monoacylglycerol lipase (MAGL), the primary hydrolytic enzyme of the endogenous cannabinoid 2-arachidonylglycerol, produce antinociceptive effects in numerous rodent models of pain, we investigated whether inhibitors of this enzyme (i.e., JZL184 and MJN110) would reverse paclitaxel-induced mechanical allodynia in mice. These drugs dose-dependently reversed allodynia with respective ED$_{50}$ values (95% C.L.) of 8.4 (5.2-13.6) and 1.8 (1.0-3.3) mg/kg. Complementary genetic and pharmacologic approaches revealed that the anti-allodynic effects of each drug require both cannabinoid receptors, CB$_1$ and CB$_2$. MJN110 reduced paclitaxel-mediated increased expression of monocyte chemoattractant protein-1 (MCP-1, CCL2) and phospho-p38 MAPK in dorsal root ganglia as well as MCP-1 in spinal dorsal horn. Whereas the antinociceptive effects of high dose JZL184 (40 mg/kg) underwent tolerance following six days of repeated dosing, repeated administration of a threshold dose (i.e., 4 mg/kg) completely reversed paclitaxel-induced allodynia. In addition, we found that the administration of MJN110 to control mice lacked intrinsic rewarding effects in the conditioned place preference (CPP) paradigm. However, it produced a CPP in paclitaxel-treated animals, suggesting a reduced paclitaxel-induced aversive state. Importantly, JZL184 did not alter the antiproliferative and apoptotic effects of paclitaxel in A549 and H460 non-small cell lung cancer cells. Taken together, these data indicate that MAGL inhibitors reverse paclitaxel-induced neuropathic pain without interfering with chemotherapeutic efficacy.
Introduction

Paclitaxel is a widely prescribed chemotherapeutic for the treatment of breast, lung and other cancers, but causes a variety of serious side effects, including peripheral neuropathy, leukopenia, joint or muscle pain, vomiting, and alopecia (Ghersi et al., 2015). Chemotherapy-induced peripheral neuropathy (CIPN) causes severe sensory disturbances that range from mild tingling to spontaneous painful burning paresthesia affecting the longest sensory nerves to the hands and feet (Dougherty et al., 2004) and can persist long after treatment cessation (Tanabe et al., 2013) in up to 68% of chemotherapy cancer patients (Seretny et al., 2014). As traditional analgesics generally lack efficacy in treating this condition (Kim et al., 2015), a pressing need exists for novel analgesic strategies. However, the endogenous cannabinoid system contains several potential targets of promise to treat CIPN. Here, we employ a mouse paclitaxel model of CIPN to explore whether inhibition of monoacylglycerol lipase (MAGL) (Dinh et al., 2002), the primary hydrolytic enzyme of the endogenous cannabinoid 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995; Blankman et al., 2007), will reduce nociceptive behavior and associated inflammatory responses.

MAGL inhibitors increase 2-AG, decrease arachidonic acid levels in the CNS (Long et al., 2009), and produce anti-allodynic effects in rodent neuropathic pain models, including chronic constriction injury of the sciatic nerve (Ignatowska-Jankowska et al., 2015; Wilkerson et al., 2016b; Crowe et al., 2017) and cisplatin neuropathy (Guindon et al., 2013). Importantly, the CB2 receptor agonist AM1710 (Deng et al., 2015b) and the MAGL inhibitor JZL184 (Slivicki et al., 2017) reverse paclitaxel-induced allodynia in mice. In the present study, we build upon this work using the MAGL inhibitors JZL184 (Long et al., 2009a) and MJN110 (Niphakis et al., 2013) in the paclitaxel mouse model of CIPN. Although MJN110 is more potent than JZL184
(Ignatowska-Jankowska et al., 2015), it also inhibits \(\alpha/\beta\)-hydrolase domain 6 (ABHD6) (Niphakis et al., 2013), which metabolizes a much smaller percentage of 2-AG than MAGL (Blankman et al., 2007). To control for this off-target effect, we tested the selective ABHD6 inhibitor KT195 (Hsu et al., 2012; Wilkerson et al., 2016a). We used complementary genetic and pharmacologic approaches to assess the contribution of CB\(_1\) and CB\(_2\) receptors to observed pharmacological effects of the inhibitors. Because CIPN persists in patients long after chemotherapy treatment, we explored whether the antinociceptive effects of JZL184 would undergo tolerance or be maintained after repeated administration. We also explore whether repeated administration of JZL184 increases 2-AG levels and decreases arachidonic acid levels, along with prostaglandin D2 (PGD\(_2\)) (Nomura et al., 2011), in the lumbar spinal cord of paclitaxel-treated mice.

In addition to neurotoxicity (Bobylev et al., 2015), paclitaxel causes inflammation within the dorsal root ganglia (DRG) and activates toll-like receptor 4 (Li et al., 2014) with subsequent p38 MAPK expression and activation through phosphorylation, increasing phosphorylated p38 MAPK (phospho-p38) (Li et al., 2015). Paclitaxel also increases the chemokine monocyte chemoattractant protein-1 (MCP-1)/(C-C motif) ligand 2 (CCL2) expression in the DRG (Zhang et al., 2013; Makker et al., 2017) and the spinal cord (Pevida et al., 2013; Zhang et al., 2013), causing sensory neuron sensitization through transient receptor potential channel activation (Jung et al., 2008) and increased intracellular calcium (Zhang et al., 2013). Inhibition of MCP-1 signaling reverses paclitaxel allodynia (Zhang et al., 2013). In contrast, inhibition of phospho-p38 does not reverse, but prevents (Li et al., 2015) allodynia development. Although a CB\(_2\) receptor agonist inhibits spinal MCP-1 expression associated with paclitaxel (Deng et al., 2015), MAGL inhibitors remain unexplored. Accordingly, we tested whether MAGL inhibition would
ameliorate paclitaxel-induced expression of MCP-1 and phospho-p38 in the DRG and spinal dorsal horn.

Along with mechanical allodynia, we assessed whether MJN110 produces a conditioned place preference (CPP) in paclitaxel-treated mice. The CPP paradigm allows for disassociation of a drug’s analgesic profile and its intrinsic rewarding effects, as shown for cisplatin neuropathy (Park et al., 2013; Krukowski et al., 2017) and osteoarthritis (Havelin et al., 2016). However, CPP has not been used in paclitaxel-induced CIPN to examine MAGL inhibition. Here, we test whether MJN110 produces a CPP in paclitaxel-treated mice, indicating relief from an aversive state not observed in control mice.

Lastly, as paclitaxel-induced CIPN develops during chemotherapy treatment, it is important to test whether candidate antinociceptive agents, such as MAGL inhibitors, interfere with paclitaxel’s anti-tumor effects. Thus, we examined whether JZL184 affects the anti-proliferative and apoptotic activity of paclitaxel in the human cell lines of non-small cell lung cancer (NSCLC) (Ettinger and Akerley, 2010) A549 (Marostica et al., 2015) and H460 (Huisman et al., 2002) cells.

Materials and Methods

Animals

Adult male C57BL/6J mice with starting weights of 24.8 ± 0.16 grams (Jackson Laboratories, Bar Harbor, ME) were used in all studies except in experiments employing CB₁ (−/−) and CB₂ (−/−) mice. Male and female CB₁ (−/−) (Zimmer et al., 1999) and CB₂ (−/−) (Jackson Laboratories, Bar Harbor, ME) and matched wild-type control mice were received from the Virginia Commonwealth University (VCU) Massey Cancer Center Transgenic/Knockout Mouse Shared Resource. Both lines of transgenic mice have been backcrossed with C57BL/6J mice.
Based on genotyping analysis, CB\(_1\) (-/-) mice were 90% similar to C57BL/6J, 6% similar to C57BL/6N and 4% similar to 129/Sv mice. CB\(_2\) (-/-) mice were 71% similar to C57BL/6J and 27% similar to C57BL/6N and 2% similar to 129/Sv mice. Animals were housed 4 per cage, with separation as needed when fighting, and maintained in the AAALAC-approved vivarium at Virginia Commonwealth University. Animals were provided with water and Teklad LM-485 Mouse Diet (7012; Envigo/Teklad Diets, Madison, WI) chow *ad libitum* with a 12-hour light/dark cycle beginning at 0600 h. Animal studies were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Institutional Animal Care and Use Committee at VCU. During studies involving transgenic mice and pharmacologic antagonists (rimonabant or SR144528), the vivarium at VCU was undergoing renovation. Thus, mice were moved between vivarium rooms and exposed to construction noise. For all other studies, mice were housed undisturbed in the vivarium. Following behavioral testing, animals were euthanized using CO\(_2\) asphyxiation and cervical dislocation unless tissue collection was performed. Tissue collection was performed after either rapid decapitation or isoflurane anesthesia.

*Assessment of Mechanical Allodynia*

Mice received a minimum of four days prior to experimentation to acclimate to the vivarium. Prior to behavioral assessment, mice were acclimated to the von Frey mesh elevated platform for a minimum of 40 min per day for a minimum of three days. During acclimation and testing, each mouse was placed in a ventilated Plexiglas cylinder, approximately 3 inches in diameter, minimizing locomotor activity. To assess paw withdrawal thresholds, von Frey filaments (North Coast Medical, Gilroy, CA) were applied to the hind paw for 3 s on each of three trials until a positive response was noted as a paw withdrawal to the stimulus. Testing
began at the 0.6 g filament and moved sequentially to higher values (1, 1.4, 2, 4 g) until 5 out of 6 withdrawals were recorded. If a response occurred at 0.6 g, the 0.4, 0.16, and 0.07 g filaments were sequentially applied until five of six positive responses were obtained. A sixth test was not conducted if the first five tests were positive. A maximum of 4 g was used to prevent sensitization. Results for each animal are reported as the average of both hind paws. In one experiment, the MJN110 time course study, a four of five threshold with a minimum withdrawal threshold of 0.6g was used only at pre-paclitaxel/vehicle baseline testing.

**Drugs and Dosing**

Paclitaxel (Taxol; Tocris Bioscience, Bristol, UK) was dissolved in a vehicle solution containing a 1:1:18 ratio of ethanol, emulphor-620 (Rhodia, Cranbury, NJ), and saline (0.9 % NaCl). A cycle of paclitaxel consisted of a total of four intraperitoneal (i.p.) injections of paclitaxel (8 mg/kg per injection) in which injections were given every other day (Toma et al., 2017). Control (no paclitaxel) mice were given four injections of vehicle. The injection volumes were 0.01 ml per gram of body mass. The Cravatt laboratory at Scripps Research Institute, La Jolla, CA synthesized MJN110 and KT195 and the Drug Supply Program at the National Institute on Drug Abuse (Bethesda, MD) provided JZL184, rimonabant, and SR1445528. Doses of JZL184 (Long et al., 2009a; Ghosh et al., 2013; Ignatowska-Jankowska et al., 2015), MJN110 (Niphakis et al., 2013; Ignatowska-Jankowska et al., 2015; Wilkerson, et al., 2016b), and KT-195 (Hsu et al., 2012; Wilkerson et al., 2016a) were selected based on the results of previous in vivo and in vitro studies using these agents.

Following basal paw withdrawal assessment, paclitaxel or corresponding vehicle was administered as described above. Paclitaxel-treated and vehicle-pretreated control mice were separately housed to prevent cross-contamination. After confirmation of paw withdrawal
thresholds, drugs were administered i.p. and mice were placed on the mesh for a minimum of 30 min prior to drug testing. Testing was conducted in a blinded manner with respect to acute drug treatment and a 72 h washout period between experiments as previously described (Ignatowska-Jankowska et al., 2015; Wilkerson et al., 2016a). All experiments were completed within eight weeks of the last paclitaxel/vehicle injection with a minimum five day washout period before switching drugs. The same cohorts of CB1 (-/-) and CB2 (-/-) and (+/+) mice were used to test MJN110 and JZL184 within five weeks following paclitaxel cessation. A one-week washout period was used before switching drugs in these animals. For time course studies, testing began 0.5 h after drug administration and was finished 24 h post-treatment. Unless otherwise indicated, the antinociceptive effects of MJN110 were tested 3 h post-administration, while those of JZL184 was tested 2 h post-treatment. Rimonabant or SR144528 was administered 30 min prior to JZL184 or MJN110 (Wilkerson, et al., 2016). Allodynia was confirmed prior to each test and persisted for eight weeks post-paclitaxel when testing was completed.

**Repeated JZL184 dosing regimen**

In this experiment, we tested whether the antinociceptive effects of JZL184 would be maintained or undergo tolerance following repeated administration. C57BL/6J mice received a cycle of paclitaxel or vehicle and 10-12 days later received repeated i.p. injections of JZL184 or vehicle as previously described (Kinsey et al., 2013). In the repeated JZL184 conditions, each mouse was given a daily i.p. injection of JZL184 (4 or 40 mg/kg) for six days. For the acute conditions, each mouse was given a daily i.p. injection of vehicle for five days and administered vehicle, 4 mg/kg, or 40 mg/kg on day 6. All mice were tested for mechanical allodynia 2 h following the final injection on day 6. This procedure is shown in Figure 16A.
**Immunohistochemistry**

Immunohistochemistry was conducted as previously described (Wilkerson *et al.*, 2012; Wilkerson *et al.*, 2016a) with slight modifications. Briefly, nine days after the cycle of paclitaxel or vehicle, mice were given an i.p. injection of MJN110 (5 mg/kg) or vehicle, and three hours later the mice were deeply anesthetized with isoflurane and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1M PBS, pH 7.4 (Thermo Fisher Scientific, Waltham, MA). Whole spinal columns were collected and fixed overnight in 4% paraformaldehyde at 4°C. Spinal columns then underwent EDTA decalcification (8% or 214.9 mM in PBS) for approximately 60 days and were sliced in 7 µm thick sections. DRG sections corresponding to the L5-L6 spinal cord were selectively examined. Tissue processing, paraffin embedding and slide preparation was provided by the Virginia Commonwealth University Cancer Mouse Models Core.

Samples were deparaffinized in Hemo De (Electron Microscopy Sciences, Hatfield, PA) and re-hydrated in 100% and 70% (v/v) ethanol (Pharmco-AAPER, Brookfield, CT) followed by PBS (Abcam, Cambridge, MA). Antigen retrieval was conducted in a pressure cooker (10 PSI) for 10 min in citrate buffer (2.1g/100mL deionized water, pH 6) followed by 10 min of cooling and 5 min in room-temperature PBS. Slides were blocked for 5-6 hours in 4.6% Normal Donkey Serum/PBS solution. Primary antibodies were applied in 0.5% bovine serum albumin/PBS solution with 1% sodium azide and incubated overnight at 4°C. The following primary antibodies were used: Rat anti-MCP-1 (1:50; ab8101; Abcam, Cambridge, MA) (Zoja *et al.*, 1997; Park *et al.*, 2011) and Rabbit anti-phospho-p38 MAPK (1:800; 4511S; Cell Signaling, Danvers, MA) (Wilkerson *et al.*, 2012b; Shi *et al.*, 2017). On the following day, slides were incubated in secondary antibodies at room temperature. For MCP-1, rhodamine red donkey anti-
rat (1:2000; 712-295-153; Jackson ImmunoResearch Laboratories, West Grove, PA) was used for two hours. For phospho-p38 MAPK, samples were incubated in biotinylated donkey anti-rabbit (1:1000 for DRG, 1:1300 for dorsal horn; 711-065-152; Jackson ImmunoResearch Laboratories, West Grove, PA) for one hour. Next, samples were incubated for one hour in streptavidin-bound biotinylated horseradish peroxidase (Ultra-Sensitive ABC Peroxidase Standard Staining Kit; 32050; ThermoFisher Scientific). This was followed by incubation with TSA Plus Cyanine 3 and Fluorescein System (PerkinElmer, Waltham, MA) with precipitation of fluorophore-labeled tyramide reagent after reaction with horseradish peroxidase. For phospho-p38 MAPK staining in the DRG and spinal dorsal horn, cyanine 3 was used as the fluorophore. For confocal microscopy, fluorescein was used to stain phospho-p38 MAPK. After fluorophore staining, slides were incubated in PBS, followed by a dip in deionized water and coverslipped using Vectashield Antifade Mounting Medium with DAPI (H-1200; Vector Laboratories, Burlingame, CA). Imaging was done at 40x with a Zeiss AxioObserver A1 inverted microscope equipped with an Axiocam MRc5 color CCD camera and ZEN 2012 software (Carl Zeiss, AG) at the Virginia Commonwealth University Microscopy Facility. Images were converted to greyscale and underwent re-thresholding to reduce background fluorescence and normalize maximum and minimum fluorescence values using Zeiss ZEN lite software (Carl Zeiss, AG) in a manner that was consistent across all sections and treatment groups for each study. Spinal cord images were rotated or inverted as necessary for consistent orientation.

Densitometry analysis was conducted as previously described (Wilkerson et al., 2012; Bagdas et al., 2016; Wilkerson et al., 2016a), according to the methods of (Samudio-Ruiz et al., 2009; Zhang et al., 2012). Densitometry analysis was conducted using Image J by selecting the anatomical location of the DRG or spinal dorsal horn in each 40x image in an unbiased manner.
for each image. For the DRG MCP-1 study, the area of analysis was selected by freehand only removing empty (black) space lacking cellular material. For the spinal dorsal horn MCP-1 study, the entire dorsal horn was selected, removing rootlets beyond the anatomical edge of the spinal cord and empty (black) space lacking cellular material. For phospho-p38 MAPK analysis in both the DRG and spinal dorsal horn, four stained nuclei were selected at random from each image with preference given to nuclei in the plane of focus. Results are reported as the average of 4 separate sections per animal minus the average of four sections from a control slide lacking primary antibody. A sample size of four animals for each treatment condition was used. Results are reported as average count/mm², taken directly from Image J, is a measure of average pixel intensity in the selected area of analysis.

For confocal microscopy and co-localization analysis, images were acquired at 63x using a Zeiss AxioObserver inverted LSM710 META confocal microscope and ZEN 2012. The entire z stack of a region was collected, and final images were generated from a single image along the z-plane.

**Lipid Quantification**

Quantification of endogenous cannabinoids was conducted as previously described (Ramesh et al., 2011; Ignatowska-Jankowska et al., 2015). Briefly, mice in the repeated administration study underwent mechanical allodynia testing and were euthanized by decapitation approximately 3-6 h after drug administration, a time point known to produce reliable elevations in 2-AG and decreased arachidonic acid (Long, et al., 2009a). Spinal cord tissues were rapidly collected by hydraulic extrusion flushing the spinal canal with saline. The spinal cord was dissected to isolate the lumbar enlargement region (L4-L6), which was immediately frozen and stored at -80°C until further processing.
On the day of lipid extraction, the pre-weighed mouse samples were homogenized with 1.4 ml chloroform:methanol (containing 0.0348 g PMSF/ml), as previously described (Kinsey et al., 2013). Six point calibration curves ranged from 0.078 pmol to 10 pmol for AEA, 0.125 nmol to 16 nmol for 2-AG, arachidonic acid and 1.75 pmol to 140 pmol PGD₂, a negative control, and blank control were prepared. ISTDs (50 µl of each of 1 pmol AEA-d₈, 1 nmol 2-AG-d₈, 1 nmol arachidonic acid-d₈ and 14 pmol PGD₂-d₄) were added to each calibrator, control and sample, except the blank control. Each calibrator, control and samples was then mixed with 0.3 ml of 0.73% w/v NaCl, vortexed, and centrifuged (10 min at 4000 × g and 4°C). The aqueous phase plus debris were collected and extracted again twice with 0.8 ml chloroform, the organic phases were pooled and organic solvents were evaporated under nitrogen gas. Dried samples were reconstituted with 0.1 ml chloroform, mixed with 1 ml cold acetone, and centrifuged (10 min at 4000 × g and 4°C) to precipitate proteins. The upper layer was collected and evaporated to dryness and reconstituted with 0.1 ml methanol and placed in auto-sample vials for analysis.

The Ultra performance liquid chromatography - tandem mass spectrometer (UPLC-MS/MS) analysis of AEA, 2-AG, arachidonic acid and PGD₂ was performed on a Sciex 6500 QTRAP system with an IonDrive Turbo V source for TurbolonSpray® (Ontario, Canada) attached to a Shimadzu UPLC system (Kyoto, Japan) controlled by Analyst software (Ontario, Canada). Chromatographic separation of AEA, 2-AG and arachidonic acid was performed on a Discovery® HS C18 Column 15cm x 2.1mm, 3µm (Supelco: Bellefonte, PA) kept at 25°C and a injection volume of 10 µL. The mobile phase consisted of A: acetonitrile and B: water with 1 g/L ammonium acetate and 0.1% formic acid. The following gradient was used: 0.0 to 2.4 minutes at 40% A, 2.5 to 6.0 minutes at 40% A, hold for 2.1 minutes at 40% A, then 8.1 to 9 min 100% A, hold at 100% A for 3.1 min and return to 40% A at 12.1 min with a flow rate was 1.0 mL/min.
The source temperature was set at 600°C and had a curtain gas at a flow rate of 30 ml/min. The ionspray voltage was 5000 V with ion source gases 1 and 2 flow rates of 60 and 50 ml/min, respectively. The mass spectrometer was run in positive ionization mode for AEA and 2-AG and in negative ionization mode for arachidonic acid, and the acquisition mode used was multiple reaction monitoring (MRM). The following transition ions (m/z) were monitored with their corresponding collection energies (eV) in parentheses: AEA: 348>62 (13) and 348>91 (60); AEA-d8: 356>63 (13); 2-AG: 379>287 (26) and 379>296 (28); 2-AG-d8: 384>287 (26); arachidonic acid: 303>259 (-25) and 303>59 (-60); arachidonic acid-d8: 311>267 (-25); PGD2: 351>271 (-23) and 351>315 (-15); PGD2-d4: 355>275 (-23). The total run time for the analytical method was 14 min. Calibration curves were analyzed with each analytical batch for each analyte. A linear regression of the ratio of the peak area counts of analyte and the corresponding deuterated ISTDs versus concentration was used to construct the calibration curves.

**Conditioned Place Preference**

An unbiased conditioned place preference paradigm was used to examine the effects of MJN110 in control mice and in paclitaxel-treated mice (Kota et al., 2007; Sanjakdar et al., 2015) and is depicted in Figure 18A. Following at least a week of acclimation to the vivarium, mice were handled for three weeks leading up to CPP conditioning. A three-chamber design was utilized (two conditioning chambers with a central acclimation chamber; ENV3013, Med Associates, St Albans, VT). The outer chambers were 20 x 20 x 20 cm with differing flooring (white mesh or black rods) and wall coloring (white or black) to distinguish each. A small grey chamber in the middle connected to each outer chamber with a door.

This experiment consisted of four groups of mice in which each group received a cycle of paclitaxel or vehicle and then received conditioning sessions in which they were either
administered vehicle each conditioning day or administered MJN110 or vehicle on alternating
days. Eight days after the final paclitaxel injection, mice were placed in the central chamber and
allowed to acclimate for 5 min. The doors to both chambers were then opened and the mouse
was allowed to explore the apparatus in a drug-free state for 15 min to record baseline chamber
preferences. Beginning on post paclitaxel day 9, half the mice in each group received a single
daily i.p. injection of vehicle or MJN110 (5 mg/kg), alternating between these treatments each
day for a total of eight conditioning sessions. MJN110 or vehicle was randomly assigned to
either the black or white chamber at the start of the experiment to avoid potential bias. The other
half of the mice received a single daily i.p. injection of vehicle prior to each conditioning
session. One hour after each injection, each mouse was placed in the appropriate conditioning
chamber for 30 min. The day after the final conditioning session, each mouse was returned to the
apparatus, but did not receive an injection, and was allowed to move freely among the chambers
for a 15 min test period. Preference scores were calculated for MJN110 treatment based on the
total amount of time spent in the MJN110-paired side (Day 10, in s) minus the baseline
preference (Day 1, in s) for the same chamber. Paclitaxel- and vehicle-treated control mice (mice
received vehicle paired to both chambers), the preference score was calculated as the average of
the test preferences minus the baseline preference for each chamber.

**Cell culture**

All lung cancer cells were maintained in DMEM supplemented with 10% (v/v) fetal
bovine serum (FB22-500HI, Serum Source International, Charlotte, NC, USA) and 1% (v/v)
combination of 10,000 U/ml penicillin and 10,000 µg/ml streptomycin (15140-122; Pen/Strep,
ThermoFisher Scientific, Carlsbad, CA). Cells were incubated at 37°C under a humidified, 5%
CO₂ atmosphere. The H460 NSCLC cell line was generously provided by the laboratory of Dr.
Richard Moran at VCU and the A549 NSCLC cell line was a gift from the laboratory of Dr. Charles Chalfant at VCU.

Paclitaxel (50nM) and JZL184 (1µM) were dissolved in DMSO, diluted with sterile PBS, and added to the medium in order to obtain the desired concentrations; less than 0.1% DMSO was present in the medium. This concentration of paclitaxel was chosen as it inhibits A549 and H460 cell colony formation in vitro (Kyte et al., 2017). This concentration of JZL184 was chosen as it inhibits MAGL in cancer cells (Nomura et al., 2010). All experiments using DMSO were performed in the dark.

Assessment of cell viability

The NSCLC cells were exposed to JZL184, paclitaxel or a combination of JZL184 and paclitaxel for 24 h, after which the drugs were removed and replaced with fresh medium. The number of viable cells was determined via trypan blue exclusion on days 1, 3, 5 and 7 post-treatment. Cells were incubated with trypsin (0.25% trypsin-EDTA) for 3 min and stained with trypan blue (15250; Invitrogen, Carlsbad, CA). The viable unstained cells were counted using a hemocytometer with bright-field microscopy.

Assessment of apoptosis

Flow cytometry analyses were performed using BD FACSCanto II (BD Biosciences, San Jose, CA) and BD FACSDiva software at the Virginia Commonwealth University Flow Cytometry Core facility. For all studies, 10,000 cells per replicate within the gated region were analyzed. When collecting samples, both adherent and floating cells were harvested with 0.1% trypsin-EDTA and neutralized with medium after 48 h of drug exposure. For quantification of apoptosis, cells were centrifuged and washed with PBS, then resuspended in 100 µl of 1x binding buffer with 5 µl of Annexin V and 5 µl of propidium iodide (556547; FITC Annexin V
Apoptosis Detection Kit; BD Biosciences, San Jose, CA). The samples were then incubated at room temperature while protected from light for 15 min. The suspension solution was then brought up to 500 µl using the 1x binding buffer and analyzed by flow cytometry.

**Statistical Analyses**

Results are reported as the mean ± S.E.M. and were prepared using GraphPad Prism 7. $ED_{50}$ values were calculated as previously described (Grim et al., 2017). Briefly, data were converted to % Maximal Positive Effect (average maximum and minimum values for drug treatment) for each cohort and plotted versus log dose values. $ED_{50}$ values and 95% confidence limits were calculated using a linear regression analysis. Potency ratios were calculated based on the distance between the two dose-response curves with 95% confidence limits. Based on the study design, a one- or two-way analysis of variance (ANOVA) was used to identify statistical differences followed by Holm-Sidak post hoc testing of significant ANOVAs. Dunnett’s test was used when comparing all treatments to a single control group in the dose-response studies. A within-subjects design was used for studies CB1 and CB2 (-/-) and (+/+), MJN110 time-course, KT-195 time-course and MJN110 antagonism studies. A repeated-measures ANOVA was used specifically for time-course studies and when comparing paw withdrawal thresholds pre- and post-paclitaxel. Unpaired $t$ tests were used to compare two groups when indicated.

Unless otherwise noted, pre-paclitaxel/vehicle baseline allodynia values were not included in statistical analysis. $n=7-8$ for all allodynia studies; $n=15-16$ per group for CPP studies; $n=4$ for immunohistochemistry studies. Grubbs’ test was used to remove any significant outliers from each group in the CPP studies only. For all experiments, the probability of a type I error ($\alpha$) was set to 5% with $P$ values of $<0.05$ considered significant.
Results

*MAGL inhibitors reverse paclitaxel-induced mechanical allodynia in dose- and time-dependent manners*

Mice undergoing a paclitaxel cycle regimen displayed significant mechanical allodynia relative to control mice within 24 h following the final injection [main interaction of drug x time F (1, 13) = 8.707, P<0.05 ; Figure 4]. Acute JZL184 (40 mg/kg, i.p.) significantly reversed paclitaxel-induced mechanical allodynia [main interaction of drug x time, F (7, 98) = 9.501, P<0.001] for up to five hours (Figure 5A). Similarly, acute MJN110 (5 mg/kg, i.p.) reversed mechanical allodynia compared to vehicle treatment [main interaction of drug x time, F (7, 98) = 6.834, P<0.001] for up to five hours post-administration (Figure 5B). In mice that did not receive paclitaxel, JZL184 (40 mg/kg) altered paw withdrawal thresholds [main interaction of drug x time, F (7, 98) = 2.204, P<0.05]; however, post hoc analysis did not reveal any significant differences compared to vehicle up to 24 h post-treatment (Figure 6A). In vehicle control mice, MJN110 (5 mg/kg) [main effect of time, P=0.48; main effect of drug, P=0.61; interaction, P=0.96] did not significantly alter withdrawal thresholds (Figure 6B). To control for ABHD6 inhibition, an off-target effect of MJN110 (Niphakis et al., 2013), KT-195 (40 mg/kg) was administered to paclitaxel- and control vehicle-treated mice. KT195 did not alter paw withdrawal thresholds in either paclitaxel-treated (P>0.3) or control animals (P>0.3) (Figure 7).

The mechanical anti-allodynic dose-response relationships of JZL184 and MJN110 from separate cohorts of mice are depicted in Figure 5C. JZL184 significantly reversed mechanical allodynia at 16 and 40 mg/kg compared to vehicle treatment [F (5, 42) = 5.74, P<0.001] within five weeks after paclitaxel treatment. JZL184 (40 mg/kg) fully reversed paclitaxel-induced allodynia and elicited similar von Frey thresholds as those from mice that did not receive
paclitaxel [t test, P>0.05]. MJN110 (3 and 5 mg/kg) significantly reversed mechanical allodynia [F (4, 35) = 4.83, P<0.01] when tested up to three weeks after a cycle of paclitaxel treatment. MJN110 (5 mg/kg) fully reversed paclitaxel-induced allodynia to withdrawal thresholds comparable to control mice not treated with paclitaxel [t test, P>0.05]. The ED₅₀ values (95% confidence limits) for JZL184 and MJN110 were 8.4 (5.2-13.6) and 1.8 (1.0-3.3) mg/kg, respectively. MJN110 was 4.7 (2.0-10.6) times more potent than JZL184.

Figure 4. Paclitaxel induces a significant mechanical alldynia. Prior to treatment with paclitaxel or vehicle, mice were assessed for paw withdrawal using von Frey filaments. After testing, 8 mg/kg of paclitaxel (or equivalent volume of vehicle) was administered i.p. on alternate days for a total of 4 injections for cumulative dose of 32 mg/kg. On the day after the last injection, paw withdrawal thresholds were assessed. Data are reported as mean ± S.E.M., n = 7-8 mice/group. ***P<0.001 vs control (vehicle-treated) mice. These mice were also included in the JZL184 time course study, Figure 5B.
Figure 5. MAGL inhibitors significantly reverse mechanical allodynia in paclitaxel-treated mice. (A) JZL184 (40 mg/kg) reverses paclitaxel-induced allodynia with maximal anti-allodynic
effects occurring from 0.5 to 5 h post administration compared to baseline (Post-Pac). (B) MJN110 reverses mechanical allodynia with maximal effects occurring 2 to 5 h post injection. Pre-Pac= baseline prior to paclitaxel (Pac) treatment. (C) JZL184 and MJN110 dose-dependently reverse mechanical allodynia in separate cohorts of paclitaxel-treated mice. Maximum reversal of allodynia was comparable to vehicle control mice treated with vehicle (Ctrl-Veh) for both drugs. Data are reported as mean ± S.E.M., n = 7-8 mice/group. *P<0.05, **P<0.01, ***P<0.001 versus vehicle-treated mice at the respective time point (A and B). Filled symbols indicate a significant effect (P<0.05) of drug versus vehicle-treated mice that received a cycle of paclitaxel (C).

Figure 6. MAGL inhibition does not enhance or depress paw withdrawal thresholds in control mice lacking paclitaxel. (A) While 40 mg/kg JZL184 altered paw withdrawal thresholds, there were no significant differences noted by post hoc testing. (B) Treatment with 5 mg/kg MJN110 did not alter paw withdrawal thresholds in control mice. Pre-Veh= baseline prior to vehicle-treatment. Post-veh = baseline prior to drug administration. Data are reported as mean ± S.E.M., n = 8 mice/group.
Figure 7. ABHD6 inhibition does not alter paw withdrawal thresholds. KT195 (40 mg/kg) did not alter paw withdrawal thresholds in either (A) paclitaxel (pac)-treated or (B) vehicle (veh) control mice. Post-Pac/Veh = baseline prior to drug administration. Data are reported as mean ± S.E.M., n = 8 mice/group.

Anti-allodynic effects of MAGL inhibitors require CB₁ receptors

A cycle of paclitaxel elicited significant mechanical allodynia in both CB₁ (+/+) and (-/-) mice [main effect of paclitaxel, F (1, 14) = 144.2, P<0.001] with no significant genotype effect [P=0.06] and no significant interaction between genotype and paclitaxel treatment (P=0.18; Figure 8A). Allodynia was stable during the four weeks of behavioral assessment. JZL184 significantly reversed paclitaxel-induced allodynia in CB₁ (+/+) mice, but not in CB₁ (-/-) mice [interaction of drug x genotype, F (1, 26) = 8.316, P<0.01; Figure 8A]. Similarly, MJN110
significantly reversed allodynia in CB1 (+/+ ) mice, but not in CB1 (-/- ) mice [interaction of drug x genotype, F (1, 28) = 5.574, P<0.05; Figure 8A].

Using a complementary pharmacologic approach, the CB1 receptor antagonist rimonabant (3 mg/kg) or vehicle was administered 30 min before each respective MAGL inhibitor. As shown in Figure 8B, rimonabant blocked the anti-allodynic effects of JZL184, but had no effects on its own [interaction of antagonist x drug, F (1, 28) = 11.56, P<0.01]. Similarly, rimonabant blocked the anti-allodynic effects of MJN110 [F (1, 28) = 40.04, P<0.001; Figure 8C]. However, rimonabant did not affect von Frey threshold in control mice that did not receive a cycle of paclitaxel [t test, P>0.05] (Figures 8B and 8C).
Figure 8. The anti-allodynic effects of MAGL inhibitors require CB₁ receptor activation. (A) A cycle of paclitaxel (Pac) leads to the development of mechanical allodynia in CB₁ (+/+) and (-/-).
mice. JZL184 (40 mg/kg) and MJN110 (5 mg/kg) significantly reverse mechanical allodynia in CB\(_1\) (+/+) mice, but not in CB\(_1\) (−/−) mice. The CB\(_1\) receptor antagonist rimonabant significantly blocks the anti-allodynic effects of 40 mg/kg JZL184 (B) and 5 mg/kg MJN110 (C) in C57BL/6J mice. Control (Ctrl) mice and vehicle (Veh) treatment groups are shown for comparison. Data are reported as mean ± S.E.M., \(n = 7\)-8 mice/group. *P<0.05 versus CB\(_1\) (+/+) mice (A). ***P<0.001 versus vehicle pre-treatment (B and C).

**Anti-allodynic effects of MAGL inhibitors require CB\(_2\) receptors**

CB\(_2\) (+/+) and (−/−) mice developed significant mechanical allodynia after a cycle of paclitaxel [main effect of paclitaxel, F (1, 14) = 113.8, P<0.001] with no difference between genotypes [P=0.35] (Figure 9A), and allodynic responses remained stable throughout the five week behavioral assessment period. JZL184 significantly reversed paclitaxel-induced mechanical allodynia in CB\(_2\) (+/+) mice, but not in CB\(_2\) (−/−) mice [interaction of drug x genotype, F (1, 28) = 21.8, P<0.001; Figure 9A]. Likewise, MJN110 reversed allodynia in CB\(_2\) (+/+) mice, but not in CB\(_2\) (−/−) mice [F (1, 28) = 9.491, P<0.01] (Figure 9A).

In the next experiment, we tested whether a CB\(_2\) receptor antagonist would block the antinociceptive effects of these MAGL inhibitors. SR144528 (3 mg/kg, i.p.) or vehicle was administered 30 min prior to JZL184 or MJN110. As shown in Figure 9B, SR144528 blocked the anti-allodynic effects of JZL184, but lacked effects on its own [interaction of antagonist x drug, F (1, 28) = 12.33, P<0.01]. Similarly, SR144528 blocked the anti-allodynic effects of MJN110 [interaction of antagonist x drug, F (1, 28) = 9.431, P<0.01; Figure 9C]. Although SR144528 produced a significant, but small reduction in paw withdrawal thresholds in control mice in the JZL184 experiment (Figure 9B), it did not affect thresholds in control mice in the MJN110 experiment (Figure 9C).
Figure 9. The anti-allodynic effects of MAGL inhibitors require CB$_2$ receptor activation. (A) A cycle of paclitaxel (Pac) leads to the development of mechanical allodynia in CB$_2$ (+/+) and (-/-) mice. JZL184 (40 mg/kg) and MJN110 (5 mg/kg) lack anti-allodynic effects in CB$_2$ (-/-) mice,
but fully reverse paclitaxel-induced mechanical allodynia in CB$_2$ (+/+) animals. The CB$_2$ receptor antagonist SR144528 blocks the anti-allodynic effects of (B) 40 mg/kg JZL184 and (C) 5 mg/kg MJN110 in C57BL/6J mice. Control (Ctrl) mice and vehicle (Veh) treatment groups are shown for comparison. Data are reported as mean ± S.E.M., n = 8 mice/group. ***P<0.001 versus CB$_2$ (+/+) mice (A). *P<0.05, ***P<0.001 versus vehicle pre-treatment (B and C).

**MJN110 attenuates MCP-1 and phospho-p38 expression in paclitaxel-treated mice**

As paclitaxel causes inflammatory responses in DRG (Li et al., 2015; Zhang et al., 2016) and spinal cord (Pevida et al., 2013; Zhang et al., 2013), we evaluated whether a MAGL inhibitor would attenuate this inflammation at 3 h, a time point corresponding to allodynia reversal. A cycle of paclitaxel led to a significant increase in MCP-1 expression in the DRG compared to mice that did not receive paclitaxel [interaction of paclitaxel treatment x MJN110 treatment, F (1, 12) = 9.426, P<0.05; post hoc: P<0.01; Figure 10A and 10B]. Treatment with MJN110 (5 mg/kg) significantly decreased MCP-1 expression in paclitaxel-treated mice [P<0.01], but not in the control vehicle-treated mice [P=1.0 for comparison between Paclitaxel-MJN110 and Control-Vehicle mice]. Paclitaxel also increased MCP-1 expression in the spinal dorsal horn, an effect that was attenuated by MJN110 pre-treatment versus vehicle [F (1, 12) = 6.294, P<0.05; post hoc P<0.01; Figure 11A and 11B]. MJN110 significantly decreased spinal MCP-1 expression in paclitaxel-treated mice to levels comparable to Control-Vehicle mice [P=0.68].

Mice given a cycle of paclitaxel also demonstrated significantly increased phospho-p38 expression in the DRG compared to control mice and this effect was inhibited by MJN110 [interaction of paclitaxel treatment x MJN110 treatment, F (1, 12) = 16.56, P<0.01; Figure 12A and 6B]. MJN110 significantly decreased DRG phospho-p38 expression in paclitaxel-treated mice to levels comparable to Control-Vehicle mice [P=0.98]. However, there were no significant changes in the expression of phospho-p38 MAPK in the spinal dorsal horn [no main effect of paclitaxel, P=0.60; no main effect of MJN110, P=0.90; Figure 13A and 13B]. Qualitative
confocal microscopy of DRG showed that MCP-1 and phospho-p38 expression co-localizes in neurons and cells consistent with the location of satellite cells as indicated by the nuclear marker DAPI, but not neuronal axons (Figure 14).
Figure 10. MJN110 attenuates paclitaxel-induced MCP-1 expression in the dorsal root ganglia. (A) Nine days after a cycle of paclitaxel, vehicle-treated mice show a significant increase of MCP-1 expression in lumbosacral dorsal root ganglia compared to control mice that did not receive paclitaxel. MJN110 significantly blocks paclitaxel-induced increases of MCP-1 expression. (B) Representative dorsal root ganglia image for each of the four treatment conditions. Scale bar = 10µm. Data are reported as mean ± S.E.M., n = 4 mice/group. **P<0.01 versus vehicle-treated mice that did not receive paclitaxel. ##P<0.01 versus vehicle-treated mice that received paclitaxel.
Figure 11. MJN110 attenuates paclitaxel-induced MCP-1 expression in the dorsal horn of the spinal cord. (A) A cycle of paclitaxel elicits a significant increase in MCP-1 expression in lumbosacral spinal cord compared to control mice that did not receive paclitaxel. MJN110 (5 mg/kg) reverses paclitaxel-induced elevations of MCP-1 expression. (B) Representative image for each of the four conditions. Scale bar = 10 μm. Data are reported as mean ± S.E.M., n = 4 mice/group. **P<0.01 versus vehicle-treated mice that did not receive paclitaxel. ###P<0.01 versus vehicle-treated mice that received paclitaxel.
Figure 12. MJN110 attenuates paclitaxel-induced phospho-p38 MAPK expression in the dorsal root ganglia. (A) Following paclitaxel treatment, vehicle-treated mice show a significant increase of phospho-p38 expression in lumbosacral dorsal root ganglia compared to control mice that did not receive paclitaxel. MJN110 significantly decreased paclitaxel-induced expression of phospho-p38. (B) Representative dorsal root ganglia image for each of the four conditions. Scale bar = 10 µm. Data are reported as mean ± S.E.M., n = 4 mice/group. ***P<0.001 versus vehicle-treated mice that did not receive paclitaxel. ###P<0.001 versus vehicle-treated mice that received paclitaxel.
Figure 13. Paclitaxel does not increase phospho-p38 expression in the spinal dorsal horn. (A) Neither paclitaxel nor MJN110 alters phosphorylated phospho-38 MAPK expression in the spinal dorsal horn. (B) Representative images for each condition. All images are at 40x. Scale bar = 10µm. Data are reported as mean ± S.E.M., n = 4 mice/group.
Figure 14. Qualitative confocal microscopy of MCP-1 and phospho-p38 MAPK co-localization in dorsal root ganglia cells. A cycle of paclitaxel (Pac) increased MCP-1 (left) and phospho-p38 (middle) expression compared to control vehicle (Ctrl) treatment. MCP-1 and phospho-p38 co-localize in cells stained with the nuclear marker DAPI (right). Thick arrows indicate neurons, and thin arrows indicate cells consistent with the location of satellite cells. This co-localization is not observed in sections from MJN110 (MJN)-treated mice compared to vehicle (Veh) treatment. All images are at 63x. Scale bar = 10µm.
Repeated administration of low-dose JZL184 prevents development of tolerance and increases 2-AG levels in the lumbar spinal cord

Treatment with paclitaxel produced stable mechanical allodynia prior to the first JZL184 or vehicle treatment [main interaction of drug x time, F (2, 92) = 35.79, P<0.001; Figure 15]. Following the treatment procedure shown in Figure 16A, the data depicted in Figure 16B show differential effects of 4 and 40 mg/kg JZL184 administered acutely or repeatedly in paclitaxel-treated mice [F (5, 42) = 8.379, P<0.001]. Acute administration of 40 mg/kg JZL184 fully reversed paclitaxel-induced allodynia [P<0.01], but this effect underwent tolerance following six days of daily administration [P=1.0]. In contrast, although acutely administered 4 mg/kg JZL184 did not significantly attenuate paclitaxel-induced allodynia [P=1.0], six days of repeated administration of this dose fully reversed the allodynia [P<0.05].

Following mechanical allodynia assessment, mice were sacrificed and the L4-L6 level of the lumbar spinal cord was procured for lipid quantification. As depicted in Figure 17A, JZL184 significantly elevated 2-AG levels [F (5, 42) = 81.37, P<0.001]. While acute [P<0.001] and repeated [P<0.001] administration of 40 mg/kg JZL184 elevated 2-AG levels, 4 mg/kg JZL184 significantly increased 2-AG spinal levels following repeated produced administration [P<0.001], but not acute administration [P=0.09]. Repeated administration of 40 mg/kg JZL184 led to higher 2-AG levels than acute treatment [P<0.001]. A significant effect was also found for spinal AEA levels [F (5, 42) = 19.25, P<0.001]. Repeated administration of either 4 mg/kg [P<0.05] or 40 mg/kg [P<0.001] JZL184 (Figure 17B) produced significantly increased spinal AEA levels. As shown in Figure 17C, arachidonic acid levels were significantly decreased [F (5, 42) = 6.238, P<0.001] following repeated administration of 40 mg/kg JZL184 [P<0.01], only. Although a significant effect was found for PGD_2 [F (5, 42) = 4.591, P<0.01], no group
significantly differed from paclitaxel control mice [P>0.1] (Figure 17D). PGD$_2$ levels significantly differed between vehicle control mice lacking paclitaxel and paclitaxel-treated mice with repeated administration of 40 mg/kg JZL184 [P<0.001]. PGD$_2$ levels also differed between acute administration of 4 mg/kg JZL184 and repeated treatment with 40 mg/kg JZL184 [P=0.03]. Compared to vehicle control mice, paclitaxel treatment did not alter 2-AG [P=0.98], AEA [P=0.95], arachidonic acid [P=0.81], or PGD$_2$ [P=0.52] levels in the lumbar spinal cord.

Figure 15. Paclitaxel (Pac)-treated mice used in the repeated administration study developed a significant mechanical allodynia compared to no paclitaxel vehicle control (Veh) mice after a cycle of paclitaxel. Allodynia was still observed prior to the first injection of JZL184 (Pre-Treatment Baseline). Data are reported as mean ± S.E.M., n = 40 paclitaxel-treated mice, n = 8 vehicle-treated mice. ***P<0.001 vs vehicle control mice.
Figure 16. Acute and repeated administration of 4 and 40 mg/kg JZL184 differentially effects on paclitaxel-induced allodynia. (A) Beginning 10-12 days after a paclitaxel cycle, JZL184 or vehicle was administered one a day for six days. On the last day, mice received a final injection of JZL184 or vehicle followed by paw withdrawal testing. (B) Although acute administration of high-dose JZL184 fully reverses paclitaxel-induced allodynia, this antinociceptive effect undergoes tolerance following six days of daily JZL184. Conversely, acute administration of low-dose JZL184 does not attenuate paclitaxel-induced allodynia, it completely reverses allodynia after repeated administration. All data were recorded two hours after the last treatment. Data are reported as mean ± S.E.M., n = 8 mice/group. *P<0.05, **P<0.01 versus the corresponding acute condition; ##P<0.01 versus no paclitaxel.
Figure 17. Acute versus repeated administration of JZL184 (4 or 40 mg/kg) on 2-AG (A), anandamide (AEA; B), arachidonic acid (C), Prostaglandin D2 (PGD2; D) in spinal cord. Data are reported as mean ± S.E.M., n = 8 mice/group. *P<0.05, **P<0.01, ***P<0.001 versus Paclitaxel-Vehicle. #P<0.05, ###P<0.001 versus the corresponding acute treatment.

**MJN110 produces a conditioned place preference in paclitaxel-treated mice**

In the next study, we examined whether a MAGL inhibitor produces CPP in vehicle-treated or paclitaxel-treated mice. Paclitaxel- and vehicle-treated mice received conditioning trials with 5 mg/kg MJN110, and were then tested without drug for preference or aversion to the MJN110-associated chamber (Figure 18A). MJN110 produced a significant place preference in paclitaxel-treated mice, but did not affect place conditioning in control mice [interaction of paclitaxel treatment x MJN110 treatment, F (1, 59) = 4.338, P<0.05; Figure 18B]. Paclitaxel-treated mice had significantly lower withdrawal thresholds than vehicle-treated animals both
before and after testing [interaction of paclitaxel treatment x time, F (2, 122) = 61.54, P<0.001; Supplemental Figure 19].

Figure 18. MJN110 leads to the development of a CPP in mice that received a cycle of paclitaxel, but not in mice that received a cycle of vehicle. The procedural schedule is shown in (A) followed by the place preference data in (B). BL = baseline preference test prior to conditioning. Test = preference test after conditioning. Data are reported as mean ± S.E.M., n = 15-16 mice/group. *P<0.05 MJN110 vs vehicle control in paclitaxel mice. Data collected with the help of Dr. Deniz Bagdas under the supervision of Dr. Imad Damaj.
Figure 19. Paclitaxel-treated mice used in the conditioned place preference (CPP) paradigm developed a significant mechanical allodynia compared to no paclitaxel vehicle control mice after paclitaxel treatment. Allodynia was still observed after CPP testing. Data are reported as mean ± S.E.M., n = 31-32 mice/group. ***P<0.001 vs vehicle control mice.

**JZL184 does not interfere with paclitaxel-induced growth arrest or apoptosis in non-small cell lung cancer cells**

The final experiment examined whether JZL184 interferes with paclitaxel-induced growth arrest or stimulates NSCLC lines. Consistent with its known anti-proliferative effects, paclitaxel decreased the number of viable A549 cells [interaction of treatment x day, F (12, 32) = 10.4, P<0.001; Figure 20A]. JZL184 did not affect viable cell number alone [P=0.94 compared to control, day 7] or in combination with paclitaxel [P=0.98 compared to paclitaxel alone; Figure 20A]. As shown in Figure 20B, paclitaxel also decreased the number of viable H460 cells [interaction of treatment x day, F (12, 32) = 115, P<0.001], and JZL184 did not affect viable cell number alone [P=0.93 compared to control, day 7] or in combination with paclitaxel [P=0.93 compared to paclitaxel alone]. Likewise, JZL184 did not affect apoptotic cell population. Paclitaxel produced apoptosis in both A549 [main effect of paclitaxel, F (1, 8)=194.4, P<0.001; Figure 21A] and H460 cells [main effect of paclitaxel, F (1, 8) = 7.549, P<0.03; Figure 21B]
when compared to control. JZL184 did not interfere with paclitaxel-induced apoptosis in either A549 [no main effect of JZL184 treatment, \(P=0.59\)] or H460 [no main effect of JZL184 treatment, \(P=0.60\)] cells.

**Figure 20.** JZL184 does not stimulate non-small cell lung cancer (NSCLC) cell proliferation alone or interfere with paclitaxel (Pac, 50nM)-induced growth inhibition of A549 (**A**) or H460 (**B**) cells. Day 0 represents the initial number of cells after seeding. JZL184 (1µM), paclitaxel or the combination of paclitaxel and JZL184 was added to the cultures on day 0 and was replaced with drug-free medium 24 hours later. The number of viable cells was determined via trypan blue exclusion. Data are expressed as the mean ± SEM of three independent experiments. **\(***P<0.001\) versus control. Data collected by Lauren Kyte and Nipa Patel under the supervision of Dr. David Gewirtz.
JZL184 does not affect paclitaxel-induced apoptosis of non-small cell lung cancer (NSCLC) cells. A549 (A) and H460 (B) cells were exposed to JZL184 (1 μM), paclitaxel (100 nM), or the combination of paclitaxel and JZL184 for 48 h. Quantification of apoptotic cells was determined using the Annexin V/PI assay, followed by flow cytometry analysis. Data are expressed as mean ± SEM of three independent experiments. Data collected by Lauren Kyte and Nipa Patel under the supervision of Dr. David Gewirtz.
Discussion

The present study replicates and extends the results of other studies showing that the MAGL inhibitor JZL184 reverses paclitaxel-induced (Slivicki et al., 2017) and cisplatin-induced (Guindon et al., 2013) allodynia. Here, we demonstrate that JZL184 and MJN110 fully reverse paclitaxel-induced mechanical allodynia with relative potencies consistent with their respective MAGL inhibitory constant estimates of 8 nM and 2.1 nM (Long et al., 2009a; Niphakis et al., 2013). Notably, neither MAGL inhibitor produced enhanced or depressed paw withdrawal thresholds in control mice lacking paclitaxel. To control for MJN110 inhibition of ABHD6, the ABHD6 inhibitor KT195 did not affect the allodynic effects of paclitaxel. The anti-allodynic effects of each MAGL inhibitor required both CB1 and CB2 receptors, as demonstrated by genetic and pharmacologic approaches. MJN110 also attenuated paclitaxel-induced increases in phospho-p38 and MCP-1/CCL2 expression, suggesting a concomitant decrease of inflammatory responses with allodynia reversal. Tolerance to the anti-allodynic effects of repeated administration, noted after a high dose of JZL184, was not observed with a subthreshold dose regimen that produced anti-allodynic effects and increased spinal 2-AG levels following repeated administration. Furthermore, MJN110 produced a CPP in paclitaxel-treated mice, but not in control animals. Lastly, we show that JZL184 treatment, at a concentration that inhibits MAGL (Nomura et al., 2010), does not interfere with the antiproliferative and apoptotic effects of paclitaxel in human cell lines of NSCLC.

Here we report that the anti-allodynic effects of MJN110 and JZL184 in the paclitaxel neuropathic pain model requires activation of both CB1 and CB2 receptors. Likewise, both receptors are required for the antinociceptive effects of MAGL inhibitors in chronic constriction injury of the sciatic nerve (Ignatowska-Jankowska et al., 2015), carrageenan (Ghosh et al., 2013).
and cisplatin (Guindon et al., 2013) models of pain. In contrast, selective CB₂ receptor agonists fully reversed paclitaxel-induced allodynia (Rahn et al., 2008; Deng et al., 2015b). Similarly, pan CB₁/CB₂ receptor agonists (CP55,940; WIN55-212; Δ⁹-tetrahydrocannabinol) reverse paclitaxel-induced alldynia in rodents (Pascual et al., 2005; Deng et al., 2015a; Deng et al., 2015b). The anti-allodynic effects of CP55,940 in paclitaxel-treated mice show decreased potency in CB₁ (-/-) mice compared with CB₂ (-/-) mice (Deng et al., 2015), demonstrating that sufficient stimulation of either receptor alone can elicit antinociceptive effects. Thus, MAGL inhibitors may require activation of both receptors because the degree to which they elevate 2-AG may be insufficient to drive anti-allodynic responses at either receptor alone. This could also be due to biased agonism at either cannabinoid receptor. At the CB₁ receptor, 2-AG increases both cAMP and ERK phosphorylation versus greater cAMP accumulation by CP55,940 (Khajehali et al., 2015). Similarly, in cells expressing CB₂ receptor, 2-AG causes greater ERK phosphorylation while cAMP accumulation is greater with CP55,940 (Shoemaker et al., 2005). Lastly, it is possible that allodynia maintenance is due to concurrent neuronal hyperexcitability (Li et al., 2017b) and inflammation (Zhang et al., 2016a). Thus, reversal of allodynia with MAGL inhibitors may require simultaneous attenuation of neuronal hyperexcitability and inflammation through activation of both cannabinoid receptors.

Paclitaxel elicits neuronal damage in DRG followed later by satellite cell hypertrophy and macrophage infiltration (Peters et al., 2007). Macrophage chemotaxis to the DRG is critical for the development of mechanical, but not cold, allodynia in the spared nerve injury model of neuropathy (Cobos et al., 2018). For paclitaxel allodynia, DRG macrophage infiltration corresponds with allodynia development (Zhang et al., 2016a). MCP-1 promotes macrophage recruitment (Zhang et al., 2016) and its expression in small nociceptive neurons sensitizes large-
and medium-sized neurons by increasing intracellular calcium (Zhang et al., 2013). Paclitaxel also increases MCP-1 expression in the spinal dorsal horn from astrocytes (Zhang et al., 2013) as well as phosphorylates p38 through toll-like receptor 4 activation in DRG neurons, but not in the spinal cord (Li et al., 2015). Phospho-p38 expression on small IB4- and CGRP-positive neurons (Li et al., 2015) leads to sodium channel activation and hyperexcitability of nociceptive neurons (Hudmon et al., 2008). Here, we confirmed that a cycle of paclitaxel induces MCP-1 expression in both the lumbosacral DRG and lumbar dorsal horn, while phospho-p38 is increased in the DRG, but not in the dorsal horn, as previously reported (Zhang et al., 2013; Li et al., 2015). As shown in Figure 14, phospho-p38 and MCP-1 co-expression occurs in the same DRG neurons and cells consistent with the location of satellite cells. The findings that MJN110 attenuates increased expression of phospho-p38 and MCP-1 from paclitaxel indicates an anti-inflammatory action, though further work is needed to determine whether CB₁ and CB₂ receptors mediate these actions. Either cannabinoid receptors may be required depending on the cell type expressing MCP-1 or phospho-p38.

Whereas prolonged and complete blockade of MAGL leads to high brain levels of endocannabinoids, CB₁ receptor down-regulation and desensitization, and tolerance to the anti-allodynic effects of MAGL inhibitors (Schlosburg et al., 2010), repeated administration of a low dose JZL184 produces elevated endocannabinoid brain levels without CB₁ receptor functional tolerance (Kinsey et al., 2013). Similarly, the present study demonstrates that anti-allodynic effects of high-dose JZL184 (40 mg/kg) in paclitaxel-treated mice undergo tolerance following repeated administration, but repeated administration of a subthreshold dose of JZL184 (4 mg/kg), which given acutely did not reverse allodynia, significantly elevated 2-AG and AEA in the L4-L6 region, and fully reversed paclitaxel-induced allodynia. MAGL also contributes to the
production of pro-inflammatory lipid mediators, such as arachidonic acid, prostanoids (Nomura et al., 2011b), and phosphatidic acids (Nomura et al., 2010), which could contribute to the antiallodynic actions of MAGL inhibitors. However, only repeated administration of high-dose JZL814 reduced arachidonic acid in the L4-L6 region of the spinal cord, and none of the JZL184 treatments significantly affected PGD\(_2\) compared with vehicle. Also, as paclitaxel leads to increased levels of lysophosphatidic acid (LPA) in the spinal cord dorsal horn and LPA receptor 1 (\(-/-\)) and 3 (\(-/-\)) mice show a phenotypic resistance to the development of paclitaxel-induced mechanical allodynia (Uchida et al., 2014), it is possible that MAGL inhibitors reduce this lipid as well as affect other mediators.

Although thermal, chemical or mechanical stimuli are widely used to assess analgesia in rodents, these outcomes may lack clinical predictive value (Mogil, 2009). Alternatively, the CPP paradigm is used to infer potential affective aspects of pain relief into rodent pain models (King et al., 2009; Navratilova and Porreca, 2014; Havelin et al., 2016). Here, we make the unique observation that the MAGL inhibitor MJN110 produces a significant place preference in paclitaxel-treated mice, but not in control mice. This pattern of findings suggests that MJN110 lacks intrinsically rewarding or aversive effects, but is rewarding in paclitaxel-treated mice. The MJN110 data from control mice are consistent with the failure of JZL184 to produce a conditioned place preference or aversion (Gamage et al., 2015). The MJN110-induced CPP in paclitaxel-treated mice may represent a relief from affective or sensory aspects of nociception, as described in rodent models of cisplatin neuropathy (Park et al., 2013; Krukowski et al., 2017). However, MAGL inhibition may also relieve other aversive states in paclitaxel-treated mice, such as a preference for the dark chamber in the light/dark box test and increased immobility time in the forced swim test (Toma et al., 2017). As MAGL inhibitors produce pharmacological
effects in laboratory animal assays used to screen antidepressant and anxiolytic drugs (Kinsey et al., 2011; Sciolino et al., 2011; Zhong et al., 2014), MJN110 chamber preference in paclitaxel-treated mice may represent relief from an aversive state distinct from neuropathy.

Because treatments for CIPN may be given while patients are still receiving chemotherapy, we tested if JZL184, at concentrations that inhibit MAGL (Nomura et al., 2010), interferes with paclitaxel-induced cell death or growth arrest. We found that paclitaxel decreases cell viability and induces apoptosis in two human cell lines of NSCLC, which were not altered by JZL184. In other cancer types (i.e. prostate, melanoma, ovarian), decreased MAGL expression or activity inhibited cell proliferation and transformation (Nomura et al., 2010; Nomura et al., 2011a), though it is unclear whether A549 and H460 cells express MAGL. Taken together, these results suggest that a MAGL inhibitor neither affects cancer growth alone nor interferes with the anti-proliferative or anti-apoptotic effects of paclitaxel in these in vitro models of NSCLC.

The results of the present study do not support the idea that the endogenous cannabinoid system contributes to the development of paclitaxel-induced allodynia. Consistent with previous findings (Deng et al., 2015a), a cycle of paclitaxel elicited sustained mechanical allodynia in both CB1 (-/-) and CB2 (-/-) mice and receptor antagonists of these receptors did not alter paclitaxel-induced allodynia. Our finding that a cycle of paclitaxel did not alter 2-AG and AEA spinal levels approximately two weeks later is consistent with those of a previous study in which paclitaxel did not alter expression of CB1 receptor, CB2 receptor, MAGL, or FAAH mRNA levels in spinal cord (Deng et al., 2015b). In contrast, cisplatin-induced CIPN leads to increased spinal 2-AG and AEA levels (Guindon et al., 2013), suggesting that the endogenous cannabinoid system differentially responds to these chemotherapeutic agents. Nonetheless, as the present
study examined only a single time point more than two weeks following paclitaxel treatment, a full time course evaluation of spinal and DRG endocannabinoid levels would be of value.

The goal of this work was to test whether MAGL inhibition reverses paclitaxel-induced allodynia as well as markers of DRG neuroinflammation. As paclitaxel neuropathies are long-lasting (Tanabe et al., 2013) substantially harm quality of life and are difficult to treat (Kim et al., 2015), novel analgesic strategies are needed. The present study demonstrates that MAGL inhibitors attenuate paclitaxel nociceptive-related behaviors using both mechanical allodynia and the conditioned place preference paradigm. We also show that MAGL inhibition ameliorates MCP-1 and phospho-p38 expression in the DRG. Taken together, the findings of the present study suggest that MAGL represents a viable target for possible treatment of CIPN.
Chapter 3: Monoacylglycerol lipase inhibitors prevent the development of paclitaxel-induced mechanical allodynia in mice
Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a serious side-effect of chemotherapy treatment affecting as many as 68% of patients (Seretny et al., 2014). In particular, the taxane paclitaxel causes burning, tingling or numbness in the hands and feet signifying sensory fiber impairment (Dougherty et al., 2004). Despite the pain and functional impacts of neuropathy, including decreased ability to work (Zanville et al., 2016), there are no therapeutics shown to prevent paclitaxel CIPN prior to onset and reduction of paclitaxel dosage remains the standard of care (Bhatnagar et al., 2014). Because paclitaxel CIPN is long-lasting and affects as many as 41% of patients three years after cessation of paclitaxel treatment (Tanabe et al., 2013), there is a pressing need for novel agents to prevent paclitaxel CIPN prior to onset.

To assess potential analgesic strategies, rodent models of paclitaxel CIPN have been developed which display enhanced sensitivity to mechanical hind paw stimulation using von Frey filaments (Polomano et al., 2001; Smith et al., 2004), analogous to the “stocking and glove” distribution of CIPN symptoms in patients (Dougherty et al., 2004). Characterization of these models reveals potential mechanisms of CIPN. In the periphery, paclitaxel causes intraepidermal nerve fiber loss (Boyette-Davis et al., 2011) and decreased sensory fiber conduction (Jamieson et al., 2007). In the DRG, paclitaxel induces monocyte chemoattractant protein 1 (MCP-1) expression (Zhang et al., 2013) and macrophage chemotaxis (Zhang et al., 2016a). In the spinal cord, paclitaxel-treated mice exhibit enhanced neuronal hypersensitivity (Cata et al., 2006) and enhanced MCP-1 expression (Pevida et al., 2013; Zhang et al., 2013) in addition to activation of astrocytes (Peters et al., 2007; Zhang et al., 2012) or microglia (Pevida et al., 2013). These pre-clinical models of paclitaxel CIPN are useful for screening candidate pharmacologic agents for possible clinical trials. One system of targets potentially capable of treating and preventing
paclitaxel CIPN is the endogenous cannabinoid system, the components of which are outlined in Chapter 1 and Figure 1.

In rodent models of paclitaxel CIPN, agents targeting various components of the endogenous cannabinoid system reverse paclitaxel-induced mechanical allodynia after onset. Mixed CB₁ and CB₂ receptor agonists (Pascual et al., 2005; Deng et al., 2015a), direct CB₂ agonists (Rahn et al., 2008; Deng et al., 2015b), and MAGL inhibitors (Slivicki et al., 2017; Chapter 2) reverse mechanical allodynia through cannabinoid receptor-mediated mechanisms. Both the CB₂ agonist AM1710 (Deng et al., 2015b) and the MAGL inhibitor MJN110 (Chapter 2) also attenuate MCP-1 expression associated with allodynia in spinal cord and DRG of paclitaxel-treated animals.

In addition to reversing paclitaxel CIPN after development, pharmacologic agents targeting the endogenous cannabinoid system prevent paclitaxel CIPN prior to onset. The mixed CB₁ and CB₂ receptor agonist WIN55,212-2 prevents mechanical allodynia development when co-administered with paclitaxel (Burgos et al., 2012; Rahn et al., 2014) in a CB₁-dependent manner. In addition to mechanical allodynia, WIN55,212-2 prevents heat hyperalgesia (Burgos et al., 2012) and cold allodynia (Rahn et al., 2014) from paclitaxel administration. When 1 mg/kg WIN55,212-2 was co-administered with 1 mg/kg paclitaxel, given on four alternate days, prevention of mechanical allodynia and heat hyperalgesia lasted for the full duration of the study, 28 days after paclitaxel cessation (Burgos et al., 2012). When 0.5 mg/kg/day WIN,212-2 was administered via osmotic minipump for 26 days, with paclitaxel administered at 2 mg/kg on four alternate days after six days of pre-treatment, mechanical allodynia prevention lasted for 14 days after paclitaxel cessation, but re-emerged over 11 days after WIN55,212-2 withdrawal (Rahn et
Cold allodynia was prevented for 14 days after paclitaxel cessation and lasted for 12 days after WIN55,212-2 withdrawal, but re-emerged at later time points.

In addition to the dual agonist WIN, direct CB2 agonists prevent paclitaxel-induced mechanical allodynia. Co-administration of the direct CB2 agonists AM1710 (Rahn et al., 2014) or MDA7 (Naguib et al., 2012) prevent mechanical allodynia development in CB2-dependent manners. Administration of 15 mg/kg MDA7 for 14 days, starting with 1 mg/kg of paclitaxel administered on four consecutive days, prevented mechanical allodynia for up to 14 days after MDA7 cessation. For AM1710 (3.2 mg/kg/day administered via osmotic minipump for 26 days, with paclitaxel administered at 2 mg/kg on four alternate days after six days of pre-treatment), mechanical allodynia prevention lasted for 14 days after paclitaxel cessation and 18 days after AM1710 withdrawal with allodynia re-emergence during later time points (Rahn et al., 2014). In addition to WIN, AM1710 and MDA7, the phytocannabinoid cannabidiol prevented paclitaxel-induced mechanical and cold allodynia in mice when co-administered with paclitaxel for over 32 days after cessation of drug treatment (Ward et al., 2011). However, this effect is CB1 and CB2-independent as co-administration of a serotonin receptor 1A antagonist, but not cannabinoid receptor antagonists, blocked prevention by cannabidiol (Ward et al., 2014). This is consistent binding studies, where cannabidiol does not activate [35S]GTPyS binding in either recombinant CB1 or CB2 receptors expressed on CHO-K1 cells (MacLennan et al., 1998).

Along with prevention of the behavioral effects of paclitaxel treatment, co-administration of WIN55,212-2 or MDA7 also prevents the development paclitaxel-induced inflammation. Both WIN55,212-2 (Burgos et al., 2012) and MDA7 (Naguib et al., 2012) co-treatment with paclitaxel reduced paclitaxel-induced astrocyte activation (GFAP expression) and microglia activation (CD11b expression) in the spinal dorsal horn 14-15 days after drug cessation. Both WIN55,212-
2 and MDA7 also reduced expression of pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) in the spinal cord (Burgos et al., 2012; Xu et al., 2014).

While inhibitors of MAGL or FAAH have not been tested for the prevention of paclitaxel CIPN, they have been evaluated in a related model of cisplatin CIPN. The MAGL inhibitor JZL184 prevents mechanical allodynia from cisplatin administration in a CB1-dependent manner when co-administered with 1 mg/kg cisplatin for seven days (Khasabova et al., 2014). Similarly, the FAAH inhibitor URB597 prevents cisplatin-induced mechanical allodynia and heat hyperalgesia in a CB1-dependent manner when co-administered with 1 mg/kg cisplatin for seven days (Khasabova et al., 2012). Neither study tested after cessation of co-administration to assess the duration of prevention or the possibility of allodynia reemergence.

Inhibition of MAGL following paclitaxel administration both reverses paclitaxel-induced mechanical allodynia and attenuates markers of inflammation, including MCP-1 expression in the DRG and spinal cord and the phosphorylation of p38 MAPK in the DRG (Chapter 2). Both of these markers are critical for allodynia development. Blockade of MCP-1 expression with intrathecal antibodies given one day before and daily during paclitaxel treatment (2 mg/kg, i.p, four alternate days) prevents the development of paclitaxel-induced mechanical allodynia as well as MCP-1-dependent macrophage accumulation in the DRG (Zhang et al., 2016a). Similarly, pharmacologic inhibition of p38 MAPK with SB203580 (10µg/day, intrathecal, beginning two days prior to paclitaxel through two days after paclitaxel, 2 mg/kg, i.p, four alternate days) partially prevents the onset of mechanical allodynia after the last paclitaxel treatment (Li et al., 2015); however, allodynia reemerged about one week after paclitaxel cessation (Li et al., 2015). Because MAGL inhibition (1) reverses both MCP-1 and phospho-p38 expression associated with paclitaxel-induced allodynia (Chapter 2), (2) prevents cisplatin-induced mechanical allodynia
(Khasabova et al., 2014), and (3) reverses mechanical allodynia from paclitaxel in CB₁- and CB₂-dependent manners (Chapter 2)—suggesting cannabinoid receptor stimulation as required for prevention of allodynia with WIN55,212-2 (Rahn et al., 2014) or direct CB₂ agonists (Naguib et al., 2012; Rahn et al., 2014)—we hypothesize that MAGL inhibitors will also prevent paclitaxel-induced mechanical allodynia.

In this study, we test the hypothesis that MAGL inhibition concurrent with paclitaxel will prevent the development of mechanical allodynia in a mouse model of paclitaxel CIPN using the selective and potent MAGL inhibitors JZL184 (Long et al., 2009a) and MJN110 (Niphakis et al., 2013). To allow for the accumulation of 2-AG before and during paclitaxel toxicity, we pre-treat mice for three days prior to paclitaxel administration, similar to the protocol used for WIN55,212-2 and AM1710 (Rahn et al., 2014), and one day after the last paclitaxel treatment. Because repeated administration of the MAGL inhibitor JZL184 at 40 mg/kg can lead to downregulation of the CB₁ receptor expression and function along with 2-AG elevation (Schlosburg et al., 2010; Kinsey et al., 2013), we utilize a low-dose treatment strategy with 4 mg/kg JZL184 that prevents tolerance development (Kinsey et al., 2013; Chapter 2). Because JZL184 also inhibits FAAH (Long et al., 2009a), elevating AEA in the brain after repeated administration of high dose JZL184 (Kinsey et al., 2013), we also validate prevention of allodynia using the more selective MAGL inhibitor MJN110 which does not inhibit FAAH (Niphakis et al., 2013). As MAGL inhibitors reverse mechanical allodynia through CB₁- and CB₂- dependent mechanisms (Chapter 2), we hypothesize that these receptors are also required for allodynia prevention and used CB₁ (+/+ and (-/-) mice to test whether CB₁ receptors mediate the prevention of paclitaxel-induced allodynia by JZL184.
Materials and Methods

Animal Subjects

Male C57BL/6J mice weighing 26.99 ± 0.43 (S.E.M.) g at the start of experimentation were obtained from either Jackson Laboratories (Bar Harbor, ME) or bred in-house at the AAALAC-approved Massey Cancer Center Transgenic/Knock-out Mouse Shared Resource at Virginia Commonwealth University (VCU, Richmond, VA). Male and female CB₁ (-/-) and (+/+) mice (Zimmer et al., 1999) were bred in-house at VCU and were approximately 95% similar to C57BL/6J mice, 3% C57BL/6N mice and 1-2% Sv/129 mice. Mice were allowed to acclimate to the vivarium for a minimum of 5 days and were given access to water and Teklad LM-485 Mouse Diet (7012; Envigo/Teklad Diets, Madison, WI) chow ad libitum. Mice were housed 4 per cage, with separation by sex and as needed due to fighting, with a 12-hour light/dark cycle beginning at 0600 h. Following testing, mice were sacrificed under isoflourane anesthesia during tissue collection.

Assessment of Mechanical Allodynia

Behavioral testing was conducted as previously described (Chapter 2) according to the schedule outlined in Figure 22. Briefly, mice were acclimated to the von Frey apparatus, consisting of an elevated screen with Plexiglas containers, approximately 3 inches in diameter to limit locomotor activity, for at least 40 minutes for four days prior to testing. Mechanical allodynia was assessed using von Frey filaments (North Coast Medical, Gilroy, CA) applied to the hind paw for 3 seconds. A positive response was recorded as a paw withdrawal to the stimulus. Withdrawal thresholds were recorded to five positive responses to six stimulus applications. For two studies utilizing JZL184, five out of five positive responses were used to determine threshold for n=2 per group in Figure 23 and n=16 in the study with CB₁ (-/-) and (+/+) mice (Figure 25). Testing
began with using the 0.6 g filament. If threshold was observed at 0.6g, the 0.07, 0.16 and 0.4 g filaments were sequentially tested until threshold was reached. If threshold was not recorded at 0.6g, the 1.0, 1.4, 2.0 and 4.0 g filaments were sequentially tested until threshold was reached.

**Drugs and Administration Protocol**

Paclitaxel (Taxol; Tocris Bioscience, Bristol, UK) was dissolved in vehicle, a 1:1:18 mixture of ethanol, emulphor-620 (Rhodia, Cranbury, NJ), and saline (0.9% NaCl). JZL184 was generously provided by the Drug Supply Program at the National Institute on Drug Abuse (Bethesda, MD) and MJN110 was synthesized by the Cravatt laboratory at Scripps Research Institute, La Jolla, CA (Niphakis et al., 2013). All drugs are administered at an injection volume of 0.01 mL per gram body mass.

One cycle of paclitaxel consists of intraperitoneal (i.p.) administration of 8 mg/kg paclitaxel on four alternate days to produce a mechanical allodynia in C57BL/6J mice (Toma et al., 2017; Chapter 2). For the prevention of mechanical allodynia (Figure 22), mice were treated with either JZL184 (4 or 40 mg/kg, i.p.) or MJN110 (5 mg/kg, i.p.) for three days prior to paclitaxel treatment. Prior to the first paclitaxel treatment, mechanical allodynia baselines (Pre-Paclitaxel) were assessed. During the cycle of paclitaxel, mice received JZL184 or MJN110 once a day and, on co-administration days, paclitaxel was administered within ten minutes of JZL184 or MJN110. On the day after the last paclitaxel treatment, mechanical allodynia was assessed (1 day post-paclitaxel) and the final injection of JZL184 or MJN110 was administered. Mechanical allodynia was assessed 2 and 19-24 h after the last drug treatment and approximately one week (8 days) and two weeks (13-15 days) after the last paclitaxel treatment.
Figure 2. Experimental design for the prevention of paclitaxel-induced mechanical allodynia. Prior to administration of paclitaxel, mice were treated with drug (4 or 40 mg/kg JZL184 or 5 mg/kg MJN110) once a day for three days. Before the first paclitaxel injection, withdrawal thresholds were assessed and mice were treated with paclitaxel within 10 minutes of drug treatment during the cycle of paclitaxel. On the day after the last paclitaxel injection, withdrawal thresholds were assessed and one final drug treatment was given. Withdrawal thresholds were assessed at 2 h, 19-24 h, one week, and two weeks following the last paclitaxel injection.

Statistical Analyses

Results are reported as the average of both left and right paws for each experiment and are shown as the mean +/- S.E.M. and prepared using Graph Pad Prism 7. A repeated-measures two-way analysis of variance (ANOVA) was used to identify main effects of drug, time and interaction of drug x time for each data set followed by Holm-Sidak post hoc testing of significant ANOVAs comparing values to the control group indicated. When indicated, unpaired t tests were used to compare two groups. For all studies n=7-8. The probability of a type I error was set to 5% with P values of less than 0.05 considered significant.

Results

MAGL inhibitors prevent the development of paclitaxel-induced mechanical allodynia

Co-administration of JZL184 (4 or 40 mg/kg, i.p.) with paclitaxel prevented the development of mechanical allodynia compared to paclitaxel-treated mice [main interaction of drug x time, F (15, 140) = 1.88, P=0.03] (Figure 23). Pre-treatment with JZL184 did not alter withdrawal thresholds compared to vehicle-treatment prior to paclitaxel administration. Treatment with one cycle of paclitaxel elicited a significant mechanical allodynia compared to vehicle control mice on one day through two weeks after the last paclitaxel injection. Concurrent treatment with 40 mg/kg JZL184 prevented allodynia development relative to paclitaxel control.
one day, one week and two weeks after the last cycle of paclitaxel as well as 2 and 19-24 h after the last JZL184 treatment. While the 4 mg/kg dose of JZL184 failed to prevent alldynia one day (P=0.6) and one week (P=0.09) after the last paclitaxel treatment, these mice recovered from alldynia two weeks post-paclitaxel when compared to paclitaxel control mice. Mice treated with 4 mg/kg JZL184 did not differ from vehicle/vehicle control mice beginning week after paclitaxel cessation (P=0.2 at one week and P=0.6 at two weeks post-paclitaxel).

**Figure 23.** JZL184 prevents the development of, or enhances recovery from, paclitaxel-induced mechanical allodynia. Co-administration of 40 mg/kg JZL184 prevents the development of paclitaxel-induced mechanical allodynia one day, one week and two weeks post-paclitaxel and 2 as well as 19-24 h post-JZL184 compared to paclitaxel control. Treatment with 4 mg/kg JZL184 failed to prevent allodynia development one day after the last paclitaxel treatment, but alldynia resolved two weeks post-paclitaxel. Data are reported as mean ± S.E.M., n = 8 mice/group. *P<0.05, **P<0.01, ***P<0.001 and written p-value vs vehicle/paclitaxel control. #P<0.05 versus vehicle/vehicle control.

Concurrent administration of 5 mg/kg MJN110 also prevented the development of paclitaxel-induced mechanical allodynia up to one week after paclitaxel [main interaction of drug x time, F (10, 105) = 2.498, P=0.01] (Figure 24). Prior to paclitaxel treatment, MJN110 did not significantly alter withdrawal thresholds compared to vehicle treatment. One cycle of paclitaxel
caused the development of mechanical allodynia compared to vehicle/vehicle control one day through two weeks post-treatment. Treatment of MJN110 with paclitaxel prevented the development of mechanical allodynia compared to paclitaxel control one day and one week post-paclitaxel as well as 2 h after the last MJN110 treatment. While prevention of allodynia was not observed compared to paclitaxel control mice two weeks after paclitaxel cessation (P=0.08), MJN110-treated mice did not differ from vehicle control mice at this time point (P=0.6). Withdrawal thresholds in MJN110-treated mice did not differ from, but tended to be elevated compared to paclitaxel control mice 24 hours after the last MJN110 treatment (P=0.1). Withdrawal thresholds in MJN110-treated mice did not differ from vehicle control at this time point (P=0.2).

Figure 24. MJN110 prevents the development of paclitaxel-induced mechanical allodynia one week after paclitaxel treatment. Co-administration of 5 mg/kg MJN110 with paclitaxel prevented the development of mechanical allodynia one day and one week post-paclitaxel as well as 2 hours after the last MJN110 treatment. Data are reported as mean ± S.E.M., n = 8 mice/group. *P<0.05, **P<0.01, ***P<0.001 and written p-values are vs vehicle/paclitaxel control.
The CB₁ receptor is not required for the prevention of mechanical allodynia with JZL184

In CB₁ (+/+) mice, a cycle of paclitaxel produced a significant mechanical allodynia compared to vehicle control, an effect that was prevented by pre-treatment with 40 mg/kg JZL184 one day through two weeks post-treatment [interaction of drug x time, F (10, 105) = 2.263, P=0.02] (Figure 25A). In CB₁ (-/-) mice, co-treatment with JZL184 prevented mechanical allodynia development compared to paclitaxel control for up to two weeks after paclitaxel treatment [interaction of drug x time, F (5, 65) = 5.126, P<0.001] (Figure 25B). Withdrawal thresholds in JZL184-treated CB₁ (-/-) mice did not differ from paclitaxel control 24 hours after the last JZL184 treatment (P=0.09); however, they did not differ from vehicle control (+/+) mice at this time point [t test, P=0.4].
Figure 25. Prevention of paclitaxel-induced mechanical allodynia is CB₁ receptor-independent. Co-administration of 40 mg/kg JZL184 prevents the development of paclitaxel induced mechanical allodynia in (A) CB₁ (+/+) and (B) CB₁ (-/-) mice compared to paclitaxel control one and two weeks after paclitaxel cessation. Both CB₁ (+/+) and CB₁ (-/-) mice were randomized and tested at the same time. Data are reported as mean ± S.E.M., n = 7-8 mice/group. **P<0.01, ***P<0.001 and written p-value vs vehicle/paclitaxel control.
Discussion

In this study, we establish that high doses of MAGL inhibitors prevent the onset of paclitaxel-induced mechanical allodynia for up to two weeks. Co-administration of the MAGL inhibitor JZL184 (high-dose; 40 mg/kg) with paclitaxel prevents the development of neuropathy up to two weeks after paclitaxel treatment. Co-administration of the low-dose (4 mg/kg) of JZL184 with paclitaxel does not prevent the initial onset of allodynia one day after paclitaxel treatment, but resolves allodynia two weeks after paclitaxel cessation. Because repeated administration of high-dose JZL184 also elevates AEA (Kinsey et al., 2013), we also tested the MAGL inhibitor MJN110. Administration of MJN110 does not elevate AEA (Niphakis et al., 2013; Ignatowska-Jankowska et al., 2015), but has a different off-target effect inhibiting ABHD6 (Niphakis et al., 2013). ABHD6 is responsible for less than 5% of 2-AG hydrolysis (Blankman et al., 2007). Here, we demonstrate that co-administration of MJN110 (5 mg/kg) also prevents paclitaxel allodynia up to one week post-administration compared to paclitaxel control. Because the anti-allodynic effects of MAGL inhibition are CB1-dependent (Chapter 2), we examined if the CB1 receptor is required for the prevention of allodynia. We show that 40 mg/kg JZL184 prevents the development of mechanical allodynia in both CB1 (-/-) and (+/+) mice, suggesting that MAGL inhibition prevents mechanical allodynia development in a CB1-independent manner. This is consistent with previous studies demonstrating that CB1 receptor expression and function is decreased in the mouse brain following repeated administration of JZL184 (Schlosburg et al., 2010; Kinsey et al., 2013), which would occur with the administration procedure used here (Figure 22). As the anti-allodynic effects of MAGL inhibition are also CB2-dependent (Chapter 2), the prevention of allodynia by JZL184 or MJN110 could be CB2-dependent. Lastly, as MAGL controls the levels of arachidonic acid and its metabolites in the
central nervous system (Nomura et al., 2011b), prevention of allodynia could also be mediated by decreased arachidonic acid metabolism.

Co-administration of 40 mg/kg JZL184 with paclitaxel prevented the development of mechanical allodynia up to two weeks after cessation of paclitaxel. Similarly, MJN110 prevented the development of allodynia up to one week post-paclitaxel, suggesting that elevated 2-AG or decreased arachidonic acid production mediates the preventative effect up to this time point. Although MJN110 did not prevent allodynia two weeks post-paclitaxel compared to paclitaxel control mice, paw withdrawal thresholds in MJN110-treated mice did not differ from vehicle control mice lacking paclitaxel at this time point. The paclitaxel control mice in this study also have higher paw withdrawal thresholds compared to other studies in this dissertation which could affect these results. Therefore, this experiment needs to be replicated to confirm full allodynia development. Prevention of allodynia with MJN110 also needs to be tested beyond two weeks post-paclitaxel to confirm the loss of protection given the lack of significant difference from vehicle control mice lacking paclitaxel. Sustained allodynia after two weeks post-paclitaxel would suggest a transient effect at this time point similar to that seen 24 h.

While MJN110 can also inhibit ABHD6 (Niphakis et al., 2013), ABHD6 is responsible for less than 5% of 2-AG hydrolysis and MAGL is responsible for more than 80% of 2-AG hydrolysis (Blankman et al., 2007). As the selective ABHD6 inhibitor KT195 fails to reverse paclitaxel-induced mechanical allodynia after onset compared to JZL184 or MJN110 (Chapter 2), we predict that ABHD6 inhibition during paclitaxel treatment would not prevent allodynia development. However, KT195 needs to be tested for the prevention of paclitaxel-induced mechanical allodynia.
While MJN110 did not prevent allodynia relative to control mice 24 h after the last MJN110 treatment, MJN110-treated mice did not differ from vehicle-treated mice lacking paclitaxel. Prevention of allodynia with MJN110 was also observed approximately one week later. As paclitaxel allodynia was prevented at 24 hours post-treatment in JZL184-treated C57BL/6J mice and CB1 (+/+ mice), but not in the CB1 (-/-) mice, we hypothesize that variability at the 24 h timepoint is due to the injection and testing procedure or a result of combined drug treatment. Stress from repeated injections, handling and testing could have a pro-allodynic effect, such as stress-induced hyperalgesia, or anti-allodynic effect as with stress-induced analgesia (Woodhams et al., 2017). In rats with spared nerve injury (SNI) surgery, chronic forced swim stress enhanced mechanical allodynia (Li et al., 2017a). However, in both CB1 (+/+) and (-/-) mice, withdrawal latencies in the hot plate test were lengthened following six minutes in the forced swim test (Valverde et al., 2000). This effect was lower following three minutes of the forced swim test, which increased hot plate withdrawal latencies in CB1 (+/+ ) mice, but did not affect withdrawal latencies in CB1 (-/-) mice (Valverde et al., 2000). As CB1 desensitization could occur following MAGL inhibition here (Schlosburg et al., 2010; Kinsey et al., 2013), stress levels from repeated injections, handling and testing conditions could affect withdrawal thresholds at the 24 h time point with differing magnitudes between mice depending on exact stimuli exposure during each experiment. Lastly, paclitaxel treatment has wide-ranging metabolic effects that could produce physiologic stress. Patients treated with paclitaxel report gastrointestinal distress, alopecia and dose-limiting myelosuppression (Legha et al., 1986). Treatment with a MAGL inhibitor along with paclitaxel and von Frey testing—causing limited food and water deprivation—could exacerbate physiologic stress at the 24 h time point causing a decrease in paw withdrawal thresholds in some mice.
While low-dose JZL184 did not prevent allodynia one day after paclitaxel cessation, allodynia resolved two weeks post-paclitaxel compared to paclitaxel control animals. This suggests that increased 2-AG and AEA levels or decreased arachidonic acid metabolism during paclitaxel treatment can enhance recovery from paclitaxel allodynia if prevention is not observed. This also suggests that higher levels of 2-AG or lower levels of arachidonic acid are needed for the initial prevention of allodynia, as repeated administration of high-dose JZL184 produces greater changes in these lipid levels in the CNS versus the low-dose treatment strategy (Kinsey et al., 2013). The mechanism underlying this effect is unknown, but may be due to protection from central sensitization, discussed further below.

Administration of MAGL inhibitors increase 2-AG levels, which can activate CB1 (Mechoulam et al., 1995) and CB2 receptors (Gonsiorek et al., 2000). However, tonic MAGL inhibition via genetic knockdown or repeated administration of a high dose JZL184 (40 mg/kg) causes decreased CB1 receptor expression and function in the brain (Schlosburg et al., 2010; Kinsey et al., 2013). CB1 receptor expression and function is spared following six days of repeated administration of low-dose (4 mg/kg) JZL184 (Kinsey et al., 2013). The observation that repeated administration of high-dose JZL184 prevented the onset of paclitaxel allodynia suggested that CB1 receptor expression and function was not required for allodynia prevention. In both CB1 (+/+) and (-/-) mice, paclitaxel caused a significant mechanical allodynia that was prevented by co-administration of high-dose JZL184 for two weeks following paclitaxel treatment. While allodynia prevention was not demonstrated in CB1 (-/-) mice 24 h after JZL184 treatment compared to paclitaxel control mice, as discussed above, they did not differ from vehicle control (+/+) mice at this time point. Prevention was also observed one and two weeks after cessation of paclitaxel treatment. This suggests that the CB1 receptor is not required for the
prevention of allodynia. Because CB$_1$ (-/-) mice display decreased motor behavior compared to (+/+) control mice (Zimmer et al., 1999), co-administration of rimonabant could also be used to test the contribution of the CB$_1$ receptor to prevention with JZL184.

Inhibition of MAGL also produces anti-inflammatory effects, such as decreased paw edema to carrageenan-induced inflammation (Ghosh et al., 2013), in a CB$_2$-dependent manner. The CB$_2$ receptor is located on cells of the immune system, including leukocytes (Bouaboula et al., 1993), macrophages (Munro et al., 1993) and microglia (Romero-Sandoval et al., 2009a), and activation of the CB$_2$ receptor reverses alldynia and markers of inflammation in rodent models of neuropathic pain (Wilkerson et al., 2012a; Deng et al., 2015b). In addition to neurotoxic effects including disruption of mitochondrial homeostasis (Bobylev et al., 2015), paclitaxel has direct pro-inflammatory effects, activating cells of the immune system through toll-like receptor 4 (TLR4) activation (Byrd-Leifer et al., 2001). This leads to increased expression of pro-inflammatory cytokines, such as MCP-1 (Zhang et al., 2013, 2016a), in the spinal dorsal horn and DRG that directly sensitizes neurons (Zhang et al., 2013) contributing to central sensitization and alldynia development. Macrophage chemotaxis secondary to MCP-1 release is also critical for alldynia maintenance (Zhang et al., 2016a). Because MAGL inhibition after paclitaxel alldynia onset attenuates MCP-1 expression in the spinal cord and DRG (Chapter 2), likely through CB$_2$ receptor activation (Deng et al., 2015b), MAGL inhibition during paclitaxel administration could provide protection from alldynia development in a CB$_2$-dependent manner. Activation of the CB$_2$ receptor secondary to MAGL inhibition during paclitaxel administration could suppress MCP-1 expression (Deng et al., 2015b), macrophage accumulation in the DRG (Montecucco et al., 2008) and microglia activation (Ma et al., 2015) in the spinal cord. These potential mechanisms would mirror prevention of paclitaxel alldynia with the direct
CB₂ agonist MDA7, which also depressed spinal microglia activation (CD11b expression) in the spinal dorsal horn. The development of central sensitization may explain why the lower dose (4 mg/kg) of JZL184 did not initially prevent allodynia, but allowed for recovery two weeks after paclitaxel cessation. Initial allodynia in mice treated with low-dose JZL184 suggests that paclitaxel may cause initial neuronal hypersensitivity (Cata et al., 2006) or inflammation (Peters et al., 2007; Li et al., 2014). However, protection from sustained inflammation in the spinal cord or DRG would prevent allodynia maintenance long after paclitaxel cessation.

In addition to CB₂-mediated mechanisms of allodynia prevention, MAGL inhibition could prevent changes in arachidonic acid. Under basal conditions, and those of neuroinflammation, MAGL serves as a rate-limiting enzyme controlling the levels of arachidonic acid and its metabolites in CNS tissues (Nomura et al., 2011b). As arachidonic acid is transformed by a variety of enzymes (cyclooxygenases, lipoxygenases, etc.) into pro-inflammatory lipids, including prostaglandins (Zeilhofer, 2007) and leukotrienes (Noguchi and Okubo, 2011), inhibition of MAGL could also decrease arachidonic acid metabolites contributing to allodynia development. However, it is unknown neuropathy development requires metabolism of 2-AG into arachidonic acid. One approach to test whether metabolism of 2-AG into arachidonic acid is required for allodynia development is to treat mice lacking DAGL α or β expression, which synthesizes 2-AG in neurons (Bisogno et al., 2006) or cells of the immune system (Hsu et al., 2012), respectively, with paclitaxel. Allodynia development in both DAGLα or β (-/-) mice compared to (+/+) controls would suggest that metabolism of 2-AG is not required for allodynia development. Lack of allodynia development in either DAGLα or β (-/-) mice would suggest that metabolism of 2-AG is required and could also suggest specific cell types mediating these effects.
While we have demonstrated that MAGL inhibition with JZL184 during paclitaxel treatment prevents mechanical allodynia development up to two weeks after paclitaxel cessation, future studies are needed using CB2 (+/+) and (-/-) mice to establish if the CB2 receptor is required for allodynia prevention. These experiments also need to be extended to establish the duration of the preventative effect with JZL184 as allodynia could re-emerge following drug cessation as indicated using MJN110. It is also important to establish the exact mechanism(s) of allodynia prevention observed here. In the periphery, MAGL inhibition may prevent intraepidermal nerve fiber loss from paclitaxel treatment (Toma et al., 2017), as seen in prevention of allodynia with other agents, such as minocycline (Boyette-Davis et al., 2011) or nicotine (Kyte et al., 2017). While the exact mechanism of neuroprotection for these agents is unknown, peripheral nerve fibers must regenerate with keratinocyte turnover in the epidermis (Gornstein and Schwarz, 2017) and fiber loss is thought be secondary to disrupted axonal transport of mitochondrial factors to the periphery (Bobylev et al., 2015). Prevention of nerve fiber loss by MAGL inhibition could be due to a protection of neuronal homeostasis in the periphery. There is also a need to examine markers of inflammation and central sensitization in the spinal cord and DRG associated with allodynia development. As previously discussed, paclitaxel induces pro-inflammatory cytokine production in the spinal cord and DRG. In the spinal cord, MAGL inhibition may prevent astrocyte activation, attenuating glutamatergic excitotoxicity (Zhang et al., 2012) and MCP-1 expression (Zhang et al., 2013) in the dorsal horn following paclitaxel administration, or microglia activation (Pevida et al., 2013) in the spinal dorsal horn. In the DRG, MAGL inhibition may prevent MCP-1 expression and macrophage accumulation associated with neuropathy (Zhang et al., 2016a). These studies could be
completed in wild-type mice and mice lacking either CB$_1$ or CB$_2$ receptors to elucidate the contribution of cannabinoid receptors to these effects.

Because paclitaxel chemotherapy causes a debilitating neuropathy that can last for years after cessation of treatment (Tanabe et al., 2013), the goal of this work was to test MAGL inhibition as potential neuroprotective strategy for later clinical development. Here we demonstrate, for the first time, that MAGL inhibition prevents mechanical allodynia from paclitaxel administration in mice. We also demonstrate a low-dose treatment strategy with JZL184, which may allow for protection from long-term neuropathy after paclitaxel cessation. In conjunction with our previous work examining MAGL inhibitors as potential analgesics for paclitaxel CIPN after neuropathy onset (Chapter 2), MAGL inhibitors need further evaluation for the prevention of paclitaxel CIPN.
Chapter 4: General Discussion

Chemotherapy-induced peripheral neuropathies are a serious side-effect in patients undergoing chemotherapy for the treatment of various cancers, including those of the lung, breast and ovary (WHO, 2017). While paclitaxel, cisplatin and vincristine are all associated with the development of peripheral sensory abnormalities, paclitaxel CIPN is particularly long-lasting, affecting as many as 41% of patients three years after chemotherapy (Tanabe et al., 2013). To date, there are no effective treatments for paclitaxel CIPN. The serotonin-norepinephrine reuptake inhibitor duloxetine can be used to treat CIPN after onset; however, the benefit is modest and there is no efficacy for taxane-induced neuropathies, including paclitaxel CIPN (Smith et al., 2013). There are also no agents capable of preventing paclitaxel CIPN prior to onset (Loprinzi, 2017). Therefore, there is a serious need for novel strategies to prevent as well as to treat CIPN. One way to screen for novel analgesic strategies is through the use of animal models, which display behaviors similar to the “stocking and glove” distribution of sensory symptoms in patients with paclitaxel CIPN (Dougherty et al., 2004). In rodent models of paclitaxel CIPN, paclitaxel produces enhanced paw withdrawal responses to mechanical, heat and cold stimulation (Polomano et al., 2001).

One potential target for the treatment and prevention of paclitaxel CIPN is the enzyme MAGL (Dinh et al., 2002), which is the primary hydrolytic enzyme degrading the endogenous
cannabinoid 2-AG (Blankman et al., 2007). Inhibition of MAGL with either JZL184 (Long et al., 2009a) or MJN110 (Niphakis et al., 2013) increases 2-AG and decreases arachidonic acid concentrations in the CNS. In the brain, MAGL is a rate-limiting enzyme for the production of arachidonic acid, where expression increases arachidonic acid levels and the levels of pro-inflammatory metabolites such as PGE$_2$ and PGD$_2$ contributing to inflammation (Nomura et al., 2011b). Following increased eicosanoid production in response to inflammatory stimuli, such as bacterial lipopolysaccharide, MAGL inhibition decreases synthesis of arachidonic acid and downstream pro-inflammatory mediators (Nomura et al., 2011b). Pro-inflammatory metabolites of arachidonic acid include leukotrienes, which are synthesized by lipoxygenases and may activate neurons causing allodynia development (Noguchi and Okubo, 2011), and prostaglandins, which are synthesized by cyclooxygenase enzymes and contribute to pain and inflammation (Zeilhofer, 2007) (see schematic in Chapter 1, Figure 1).

In rodent models of CIPN, JZL184 treatment reverses mechanical allodynia after development from paclitaxel (Slivicki et al., 2017) or cisplatin (Guindon et al., 2013) administration. Reversal of cisplatin-induced mechanical allodynia with JZL184 is mediated by both CB$_1$ and CB$_2$ receptors, suggesting that stimulation of these receptors by 2-AG depresses allodynia. In rodent models of neuropathic pain, such as chronic constriction injury of the sciatic nerve, inhibition of MAGL with JZL184 reverses enhanced paw withdrawal responses to mechanical, heat and cold stimuli (Kinsey et al., 2009; Schlosburg et al., 2010; Ignatowska-Jankowska et al., 2015). These effects are also mediated by the cannabinoid receptors CB$_1$ and CB$_2$ in a CCI model with bilateral allodynia (Ignatowska-Jankowska et al., 2015) and was CB$_1$-, but not CB$_2$-, mediated in CCI models with unilateral allodynia (Kinsey et al., 2009, 2010). In addition to their antinociceptive effects in rodent models of pain, MAGL inhibitors exhibit anti-
inflammatory effects through both cannabinoid receptor-dependent and -independent mechanisms through CB$_2$ receptor activation or decreased arachidonic acid production, respectively. In the carrageenan model of inflammatory pain, administration of JZL184 decreases both mechanical allodynia and paw edema (Ghosh et al., 2013). While the anti-allodynic effects of JZL184 in this model required both CB$_1$ and CB$_2$ receptors, the reversal of paw edema was CB$_2$-dependent (Ghosh et al., 2013). As the CB$_2$ receptor is found on macrophages (Munro et al., 1993) and leukocytes (Galiegue et al., 1995), suppression of paw edema in a CB$_2$-dependent manner suggest decreased recruitment to or activation of cells of the immune system in the carrageenan-treated paw (Ghosh et al., 2013). This is consistent with findings *in vitro* where treatment with the CB$_2$ receptor agonist JWH015 suppresses both macrophage chemotaxis to MCP-1 and expression of Intercellular Adhesion Molecule 1 (ICAM-1) critical for extravasation (Montecucco et al., 2008).

As paclitaxel neuropathies develop as a result of combined neurotoxic and inflammatory mechanisms (Zhang et al., 2012; Bobylev et al., 2015), we hypothesize that MAGL inhibition will alleviate and prevent mouse paclitaxel-induced mechanical allodynia through cannabinoid receptor mechanisms of action. Supporting this hypothesis, we demonstrate that MAGL inhibition reverses paclitaxel-induced mechanical allodynia through a mechanism of action requiring both cannabinoid receptors. We also demonstrate that MAGL inhibition reverses markers of paclitaxel-induced inflammation in dorsal root ganglia and spinal cord. Using the conditioned place preference paradigm, we establish that MAGL inhibition alleviates a paclitaxel-induced affective state not present in control mice lacking paclitaxel. We also show that co-administration of JZL184 and paclitaxel does not interfere with the anti-proliferative or anti-apoptotic effects of paclitaxel in A549 or H460 non-small cell lung cancer cell lines. Finally,
we establish that MAGL inhibition during paclitaxel treatment prevents the development of mechanical allodynia in the mouse model of paclitaxel CIPN.

*Monoacylglycerol lipase inhibitors reverse mechanical allodynia in a mouse model of paclitaxel CIPN*

Intraperitoneal administration of 8 mg/kg paclitaxel every other day produced a significant mechanical allodynia compared to vehicle treatment in C57BL/6J mice as assessed using von Frey filaments and consistent with previous studies using this same paclitaxel administration protocol (Donvito et al., 2016; King et al., 2017; Toma et al., 2017). Administration of JZL184 or MJN110 significantly reversed mechanical allodynia in paclitaxel-treated mice. Maximum anti-allodynic effects of both inhibitors were comparable to pre-paclitaxel baselines, indicating full allodynia reversal. As neither MAGL inhibitor elevated or depressed withdrawal thresholds in control vehicle-treated mice, MAGL inhibition only produced anti-allodynic effects in paclitaxel-treated mice. However, our results here are consistent with control mice from a study assessing CCI-induced allodynia where neither MJN110 or JZL184 increased paw withdrawal thresholds in mice following sham surgery (Ignatowska-Jankowska et al., 2015).

For both MAGL inhibitors, significant anti-allodynic effects lasted for 5 hours post-administration. This differs from the duration of MAGL inhibition in the mouse brain, which lasts for at least eight hours post-treatment with either MAGL inhibitor as determined using activity-based protein profiling (Long et al., 2009a; Niphakis et al., 2013). MJN110 also demonstrates a shorter duration of action in the CCI model of neuropathy compared to brain MAGL inhibition, with anti-allodynic effects lasting for three hours post-treatment (Ignatowska-Jankowska et al., 2015). As paclitaxel- (Chapter 2) and CCI- (Wilkerson et al., 2012a, 2012b)
induced allodynia are associated with inflammatory changes in both the spinal cord and DRG, MAGL inhibition at sites outside of the brain may mediate the anti-allodynic effects of both MAGL inhibitors. Thus, the duration of MAGL inhibition by MJN110 and JZL184 in the spinal cord and dorsal root ganglia needs to be assessed using activity-based protein profiling and measurement of endocannabinoid levels. The duration of anti-allodynic effects could differ between paclitaxel- and CCI-induced neuropathies due to differences in MAGL expression between models. MAGL expression is increased in the spinal dorsal horn in mice with the CCI model of neuropathy as determined using immunohistochemistry (Wilkerson et al., 2012a), while there is no change in spinal MAGL mRNA levels following paclitaxel treatment (Deng et al., 2015b). Following irreversible inhibition with JZL184 or MJN110, increased production of MAGL in the spinal cord of mice with CCI surgery could reduce 2-AG levels at a faster rate than in paclitaxel-treated mice, shortening the length of anti-allodynic effects. Because inflammation in the DRG can also mediate paclitaxel allodynia (Zhang et al., 2016a), MAGL expression also needs to be assessed in the DRG of paclitaxel-treated mice. Lastly, it is important to note that there could also be pharmacokinetic differences between models due to paclitaxel treatment. Paclitaxel treatment can delay vascular healing and homeostasis in response to trauma (Radke et al., 2011), which could alter MAGL inhibitor distribution and clearance compared to mice lacking paclitaxel.

In the dose-response study, both MJN110 and JZL184 fully reversed mechanical allodynia in dose-dependent manners compared to vehicle treatment. MJN110 is approximately four-fold more potent than JZL184, as expected based on their respective MAGL inhibition constants in isolated mouse brain proteomes: 2.1 nM for MJN110 (Niphakis et al., 2013) and 8 nM for JZL184 (Long et al., 2009a). This is similar to previous results using JZL184 (Slivicki et
In the study by Slivicki, et al., administration of paclitaxel, 4 mg/kg, i.p. for four alternate days to mice produced a significant mechanical allodynia as assessed using an electric von Frey apparatus (Slivicki et al., 2017). Administration of JZL184 dose-dependently reversed paclitaxel alldynia with an ED$_{50}$ of approximately 9 mg/kg (Slivicki et al., 2017), which is comparable to the ED$_{50}$ of 8.4 mg/kg for JZL184 reported here.

Lastly, our approach controls for known off-target effects of either MAGL inhibitor. While JZL184 can inhibit FAAH (Long et al., 2009a), which is anti-allodynic in paclitaxel-treated mice (Slivicki et al., 2017), MJN110 does not inhibit FAAH (Niphakis et al., 2013). To control for the off-target effect of ABHD6 inhibition by MJN110 (Niphakis et al., 2013), we utilized the selective ABHD6 inhibitor KT195 (Hsu et al., 2012), which did not affect paw withdrawal thresholds in paclitaxel-treated mice. This suggests that the anti-allodynic effects of MJN110 treatment are independent of ABHD6 inhibition. Taken together, reversal of allodynia by JZL184 or MJN110, with in vivo potencies comparable to MAGL inhibition constants, suggests that the anti-allodynic effects of acute JZL184 or MJN110 treatment here are due to inhibition of MAGL.

**The anti-allodynic effects of monoacylglycerol lipase inhibitors are CB$_1$- and CB$_2$-dependent**

To test the hypothesis that the cannabinoid receptors mediate the anti-allodynic effects of JZL184 and MJN110, we employed genetic and pharmacologic approaches. Administration of paclitaxel to CB$_1$ (-/-) and CB$_2$ (-/-) and matched (+/+) control mice induced a significant mechanical allodynia in all groups compared to pre-paclitaxel paw withdrawal thresholds consistent with the report of Deng and colleagues (Deng et al., 2015a). Both CB$_1$ (-/-) and CB$_2$ (-/-) mice developed mechanical allodynia following paclitaxel treatment, suggesting that allodynia development—involveing spinal and DRG inflammation as described in Chapter 1—is
cannabinoid receptor-independent. This is consistent with a lack of change in endogenous cannabinoid levels in the spinal cord from paclitaxel treatment (Chapter 2); however, this was only assessed at one time point. It is also important to note that the mechanism of alldynia development may differ between (-/-) and (+/+) controls. CB1 (-/-) mice exhibit CNS defects as proper growth cone development and neuronal connectivity in the cortex is regulated by endogenous cannabinoids activating the neuronal CB1 receptor (Berghuis et al., 2007). We demonstrate that the anti-allodynic effects of JZL184 and MJN110 require both cannabinoid receptors as neither CB1 (-/-) mice nor CB2 (-/-) mice demonstrated anti-allodynic effects following MAGL inhibition compared to (+/+) control mice. These results mirrored our findings using complementary approaches with CB1 and CB2 receptor antagonists in C57BL/6J mice. Pretreatment with either the CB1 receptor antagonist rimonabant or the CB2 receptor antagonist SR144528 blocked the anti-allodynic effects of both JZL184 and MJN110. These results support our hypothesis that the anti-allodynic effects of MAGL inhibition require both cannabinoid receptors.

Agonists targeting both receptors and CB2-selective agonists (summarized in Chapter 1, Table 1) reverse paclitaxel-induced mechanical allodynia; however, stimulation of either receptor alone by 2-AG is insufficient to reverse allodynia. This suggests that the anti-allodynic effects of MAGL inhibitors requires concurrent activation of both receptors which may be due to the contributions of both neurons and cells of the immune system to allodynia maintenance. As the CB1 receptor is most highly expressed on neurons (Devane et al., 1988; Hohmann and Herkenham, 1999), which could depress paclitaxel-induced neuronal hypersensitivity (Cata et al., 2006), neuronal hypersensitivity may be sustained in mice lacking CB1 expression or function with MAGL inhibition. In contrast, the CB2 receptor is highly expressed on
macrophages (Munro et al., 1993), which accumulate in the DRG following paclitaxel treatment (Zhang et al., 2016a), and microglia (Romero-Sandoval et al., 2009b), which are activated following paclitaxel administration (Pevida et al., 2013). As activation of the CB2 receptor could attenuate both macrophage activation in the DRG (Montecucco et al., 2008) and microglia activation (Romero-Sandoval et al., 2009b) in the spinal dorsal horn, inflammation promoting neuronal hypersensitivity may be sustained in mice lacking CB2 expression or function with MAGL inhibition. As the doses of JZL184 and MJN110 used here substantially elevate 2-AG in the CNS (Long et al., 2009a; Niphakis et al., 2013) and 2-AG is a full agonist at both the CB1 and CB2 receptors (Sugiura et al., 1996; Hillard, 2000) when compared to WIN55,212-2, further increases in 2-AG levels are unlikely to produce anti-allodynic effects in mice lacking CB1 or CB2 expression or function. The requirement for both cannabinoid receptors compared to direct agonists acting via one or both receptors (Chapter 1, table 1) could also be due to biased agonism at either receptor. 2-AG increases both cAMP and ERK phosphorylation via the CB1 receptor while cAMP accumulation is greater than ERK phosphorylation for CP55,940, WIN55,212-2 and THC (Khajehali et al., 2015). For the CB2 receptor, 2-AG favors ERK phosphorylation while cAMP accumulation is greater than ERK phosphorylation for CP55,940 (Shoemaker et al., 2005).

Low-dose JZL184 produces anti-allodynic effects with repeated treatment and elevates spinal 2-AG levels

As treatment with a MAGL inhibitor following onset of paclitaxel CIPN would require repeated dosing, it is important that pharmacologic agents avoid tolerance development in pre-clinical models of neuropathy. Chronic inhibition of MAGL, whether by genetic knockout (Schlosburg et al., 2010) or repeated administration of JZL184 (Schlosburg et al., 2010; Kinsey
et al., 2013), decreases CB$_1$ receptor expression and function in the brain (Schlosburg et al., 2010; Kinsey et al., 2013). In the CCI model of neuropathy, repeated administration of high-dose (40 mg/kg) JZL184 causes loss of the anti-allodynic effects of JZL184 (Schlosburg et al., 2010; Kinsey et al., 2013). However, repeated administration of low-dose (4 mg/kg) JZL184 retained both anti-allodynic effects and CB$_1$ function (Kinsey et al., 2013). As the CB$_1$ receptor is required for the anti-allodynic effects of both JZL184 and MJN110 in the paclitaxel model of neuropathy, we predict that repeated administration of a MAGL inhibitor would produce tolerance to its anti-allodynic effects. Administration of 40 mg/kg JZL184 once a day for six consecutive days produced tolerance to the anti-allodynic effects observed with acute administration. This effect is likely due to decreased CB$_1$ expression and function, consistent with the requirement of the CB$_1$ receptor demonstrated using both genetic and pharmacologic approaches. To circumvent tolerance development, we predicted that administration of low-dose (4 mg/kg) JZL184 over six days would cause the development of anti-allodynic effects by increasing 2-AG levels in the CNS. While acute administration of 4 mg/kg JZL184 did not significantly reverse mechanical allodynia in the dose-response study, repeated administration of this dose for six days produced anti-allodynic effects.

Following assessment of mechanical allodynia, mice were sacrificed and endocannabinoid levels were assessed in the whole lumbar spinal cord. 2-AG levels were significantly elevated following acute and repeated administration of high-dose JZL184 as well as repeated administration of low-dose JZL184 compared to vehicle treatment in paclitaxel-treated mice. Acute treatment with low-dose JZL184 did not significantly elevate 2-AG levels in the lumbar spinal cord while repeated administration of high-dose JZL184 produced the greatest elevations in 2-AG. This pattern of findings corresponds with the anti-allodynic effects of
JZL184 which appear to be due to increased 2-AG levels.

Tolerance to the anti-allodynic effects of repeated administration of high-dose JZL184 is likely due to down-regulation of the CB₁ receptor as reported in the brain of mice following the same treatment procedure (Kinsey et al., 2013). In a study with a longer treatment period, repeated administration of 40 mg/kg JZL184 for 14 days increased 2-AG levels and decreased CB₁ receptor function in the hippocampus while treatment with 4 mg/kg JZL184 did not decrease CB₁ receptor function in the hippocampus (Feliszek et al., 2016). However, 2-AG levels were not elevated following repeated administration of 4 mg/kg JZL184 for 14 days in the hippocampus (Feliszek et al., 2016) suggesting differential regulation of 2-AG levels in different regions of the CNS. While it is also possible that decreased CB₂ receptor expression and function could occur following repeated administration of JZL184, the anti-allodynic effects of a direct CB₂ agonist, AM1710, are spared with eight days of repeated administration in a mouse model of paclitaxel CIPN (Deng et al., 2015b). Furthermore, in MAGL (-/-) mice, there is no decrease in CB₂ mRNA expression compared to (+/+ ) control mice (Chanda et al., 2010). As MJN110 also increases 2-AG levels in the CNS (Niphakis et al., 2013), we predict that repeated administration of high-dose MJN110 would also produce loss of its acute anti-allodynic effects.

The impact of JZL184 treatment on anandamide, arachidonic acid and other spinal lipids

In addition to increased 2-AG levels, repeated administration with either 4 or 40 mg/kg JZL184 significantly elevated AEA levels compared to vehicle in paclitaxel-treated mice. The anti-allodynic effects of repeated administration of 4 mg/kg JZL184 could be due to increased AEA levels, as FAAH inhibition produces anti-allodynic effects in paclitaxel-treated mice (Slivicki et al., 2017). However, inhibition of FAAH with a peripherally-restricted inhibitor produces anti-allodynic effects in mice following CCI surgery (Clapper et al., 2010), suggesting
that the anti-allodynic effects of FAAH inhibition may occur outside of the spinal cord and brain.

Paclitaxel treatment did not significantly alter 2-AG or AEA levels compared to vehicle control 15-17 days after paclitaxel cessation. This is consistent with the report by Deng, et al. (Deng et al., 2015b), which found that paclitaxel treatment did not alter mRNA expression of MAGL or FAAH in the spinal cord 15 days after the start of paclitaxel treatment. In contrast, cisplatin treatment increases 2-AG and AEA levels in the lumbar spinal cord 16 days after the start of cisplatin treatment (Guindon et al., 2013). These changes could be due to differential mechanism of neuropathy between models as paclitaxel induces spinal astrocyte (GFAP expression) activation or microglia (Iba-1 expression) activation not induced by cisplatin treatment (Park et al., 2013). However, these interpretations must not be overstated as observations in paclitaxel- and cisplatin-treated mice are typically limited to selected time points after chemotherapy initiation. Larger studies establishing a time course of neuropathy development with respect to markers of inflammation and lipid level changes in the spinal cord and DRG are needed.

Surprisingly, inhibition of MAGL with 4 or 40 mg/kg JZL184 did not significantly reduce arachidonic acid levels in the lumbar spinal cord compared to vehicle treatment 15-17 days after paclitaxel cessation. Similarly, levels of PGD\textsubscript{2} did not differ with JZL184 treatment compared to vehicle in paclitaxel-treated mice. This suggests that decreased arachidonic acid and PGD\textsubscript{2} levels in the spinal cord following acute administration of 40 mg/kg JZL184 and repeated administration of 4 mg/kg JZL184 are not responsible for the observed anti-allodynic effects. In the brain, acute or repeated administration of 4 or 40 mg/kg JZL184 significantly reduces arachidonic acid levels (Kinsey et al., 2013), which differs from the decreased levels seen here only with repeated administration of 40 mg/kg JZL184. This suggests tissue-specific regulation
of arachidonic acid levels, which could also be controlled by cytosolic and calcium-independent phospholipase A2 in the spinal cord of paclitaxel-treated mice (Uchida et al., 2014). Paclitaxel treatment did not alter arachidonic acid or PGD$_2$ levels in the lumbar spinal cord compared to vehicle control mice lacking paclitaxel. These results are consistent with the clinical failure of NSAIDS to effectively control pain from paclitaxel CIPN (Kim et al., 2015) by inhibiting prostaglandin production from arachidonic acid (Zeilhofer, 2007). In mice, the cyclooxygenase inhibitors indomethacin, diclofenac and celecoxib do not reverse paclitaxel-induced mechanical allodynia while one inhibitor, etodolac, reverses paclitaxel allodynia in a cyclooxygenase-independent manner (Ito et al., 2012). Conversely, reduced production of other arachidonic acid metabolites—such as leukotrienes or thromboxanes—may play a role as these lipid levels could be elevated without a measured increase in arachidonic acid levels due to rapid metabolism (Nomura et al., 2011b). Lastly, MAGL also controls the levels of free fatty acids in cancer cells, including lysophosphatidic acid (Nomura et al., 2010), which is increased in the spinal cord of paclitaxel-treated mice (Uchida et al., 2014). As LPA receptor activation is required for paclitaxel allodynia development, inhibition of MAGL may produce anti-allodynic effects by decreasing the production of LPA or similar free fatty acids (Uchida et al., 2014). Reduced production of lipid mediators derived from 2-AG is also observed with DAGL$\beta$ inhibition, which decreases 2-AG levels and metabolism in macrophages (Hsu et al., 2012). Inhibition of DAGL$\beta$ reverses paclitaxel-induced mechanical allodynia (Wilkerson et al., 2016a) suggesting that reduced 2-AG metabolism may also contribute to the anti-allodynic effects of MAGL inhibitors.

**Limitations of mechanical allodynia as a measure of paclitaxel CIPN**

The use of animal models to simulate pain states reported by humans is limited by differences between species in addition to the limitations of behavioral testing. While stimulus-
withdrawal behaviors can be objectively scored in rodents, enhanced withdrawal responses could represent reflexive responses, which are mediated at the spinal level with inputs from higher spinal levels (Sandrini et al., 2005). Thus withdrawal responses can lack clinical face validity as patients with chronic pain often report spontaneous pain and may not withdraw from painful stimuli in a reflexive manner (Mogil, 2009). Furthermore, positive “analgesic” results in animal models of withdrawal behaviors do not always translate into humans (Mogil, 2009). For example, gabapentin reverses both paclitaxel- (Donvito et al., 2016) and cisplatin- (Park et al., 2013) induced mechanical allodynia, but is not recommended clinically to treat CIPN (Kim et al., 2015; Loprinzi, 2017). This has led to an expansion in endpoints used to assess antinociceptive effects in rodents models, including restoration of pain-depressed innate behaviors such as nest building (Negus et al., 2015) or paw contact with flooring (Huehnchen et al., 2013). Another approach is the conditioned place preference paradigm with a CPP indicating possible analgesic effects, as introduced in Chapter 1 and discussed later in this section.

In rodent models of paclitaxel CIPN, paclitaxel produces enhanced hind paw withdrawal responses to mechanical, thermal and cold stimulation (Polomano et al., 2001). While animal models of paclitaxel CIPN are used to screen novel analgesics for therapeutic development, differences in neuropathy development between animals and humans remain problematic. In patients with paclitaxel CIPN, neuropathy development starts in the fingers or toes, spreading to the palm or sole, with altered sensation extending to the wrist or ankle (Dougherty et al., 2004). When asked to describe the sensory alterations in their hands and feet, 100% of patients chose the word “numb”, followed by “tingling” (81.8%), “cold” (63.6%) and “burning” (54.6%) to describe symptoms of pain (Dougherty et al., 2004). Touch detection thresholds in the hands of paclitaxel-treated patients was elevated compared to control subjects as assessed using von Frey
filaments, indicating that tactile discrimination is decreased in patients receiving paclitaxel (Dougherty et al., 2004). Similarly, detection of sharp stimuli was decreased, with sharp stimuli reported as innocuous touch in patients with paclitaxel CIPN (Dougherty et al., 2004). These findings in humans are in contrast to mice, which exhibit enhanced withdrawal responses to mechanical stimuli as assessed using von Frey filaments (Chapter 2; Polomano et al., 2001). In patients receiving paclitaxel, impaired touch and sharp sensation is attributed to dysfunctional Aβ and Aδ fiber function, respectively (Dougherty et al., 2004). In mice receiving paclitaxel, both Aβ and Aδ fibers exhibit hypersensitization with lower paw withdrawal thresholds to electric stimuli preferentially activating Aβ or Aδ fibers (Matsumoto et al., 2006). Despite this difference in mechanical sensitivity between mice and humans, alterations in cold sensitivity may be shared. Patients with paclitaxel CIPN were more sensitive to cold stimuli with an increase in pain perception compared to control subjects (Dougherty et al., 2004). Similarly, paclitaxel-treated mice exhibit cold allodynia (Smith et al., 2004).

Differences between mouse and human responses to paclitaxel treatment could be due to a variety of factors including paclitaxel dose and route of administration as well as genetic factors. Not all mice receiving paclitaxel develop mechanical allodynia and mechanical sensitivity is strain-dependent (Smith et al., 2004). Following paclitaxel treatment, C57BL/10J mice do not develop mechanical allodynia while DBA/2J mice develop enhanced mechanical sensitivity compared to C57BL/6J mice (Smith et al., 2004). Similarly, not all patients receiving paclitaxel develop the symptoms of CIPN with the likelihood of neuropathy development linked to single nucleotide polymorphisms (Leandro-García et al., 2013) and differing between races, with African American patients at greater risk (Bhatnagar et al., 2014).

Despite challenges in translation, mechanical allodynia is a widely-used measure of
paclitaxel CIPN in rodent models (Polomano et al., 2001; Smith et al., 2004). One reason for this is the correlation between behavioral endpoints and markers of neuropathy and inflammation. In mice treated with paclitaxel, allodynia development corresponds with markers of inflammation in the spinal cord (Zhang et al., 2012), DRG (Zhang et al., 2016a) and periphery (Boyette-Davis et al., 2011) as discussed in Chapter 1. As experiments correlating neuropathy development with specific markers of inflammation are difficult to conduct in human patients, rodent models of neuropathy allow for the identification of mechanisms underlying neuropathy development and maintenance in a controlled manner.

Along with data from behavioral endpoints, attenuation of markers of inflammation could also allow for improved endpoints in clinical trial design beyond pain questionnaires (Gregory et al., 2013). For example, paclitaxel CIPN causes loss intraepidermal nerve fibers in rodent models (Boyette-Davis et al., 2011; Toma et al., 2017) as well as C-fiber dysfunction (Xiao and Bennett, 2008). In humans, paclitaxel treatment decreases electrochemical skin conductance, thought to reflect changes in C-fiber sweat gland innervation (Saad et al., 2016). Surprisingly, these physiologic changes have poor correlation with a clinical pain questionnaire used to assess neuropathy (Saad et al., 2016), suggesting that pain questionnaires may not always reflect neuropathy development. Inclusion of electrochemical skin conductance as an endpoint in clinical trials may allow for the identification of novel therapeutics capable of preventing or treating paclitaxel CIPN. These endpoints could also allow for detection of neuropathy prior to symptom development, which can inform clinical decision-making and treatment plans.

**MJN110 produces a conditioned place preference in paclitaxel-treated mice**

Due to the potential limitations of mechanical allodynia as a measure of paclitaxel CIPN in mice, as described above, we employed the CPP paradigm to assess potential antinociceptive
effects of MAGL inhibition. In addition to sensory symptoms of neuropathy, paclitaxel treatment causes functional impairments, including decreased ability to work (Zanville et al., 2016), and sensory symptoms from many types of CIPN cause depression, anxiety and reduced quality of sleep (Hong et al., 2014). Successful analgesic therapy should not only relieve the nociceptive components of neuropathy, but also potential emotional/affective impacts, which are mediated by the nucleus accumbens and amygdala (Navratilova and Porreca, 2014). The conditioned place preference paradigm is used to incorporate these emotional/affective states into rodent models of CIPN when screening candidate analgesics. In rodent models of CIPN, candidate analgesic treatment is paired with a chamber while control/placebo treatment is paired with the opposite chamber. After conditioning, rodents are allowed to explore the CPP apparatus in a drug-free state with an increased time spent in the drug-associated chamber indicative of a place preference. In rodents treated with cisplatin, treatment with gabapentin, which reversed mechanical allodynia, produced a significant place preference for the gabapentin-associated chamber (Park et al., 2013). As no place preference developed for either chamber in control mice that did not receive cisplatin, preference for the gabapentin-associated chamber was interpreted as relief from the neuropathic pain of cisplatin CIPN (Park et al., 2013). While gabapentin is not recommended for the treatment of cisplatin CIPN (Kim et al., 2015; Loprinzi, 2017), gabapentin is recommended for the treatment of other types of neuropathic pain (Finnerup et al., 2015). This same approach was applied to a novel candidate analgesic, a histone deacetylase inhibitor, with a place preference for the drug-associated chamber in cisplatin-treated mice interpreted as relief from CIPN symptoms (Krukowski et al., 2017). As this approach was successfully employed in the cisplatin model of CIPN to screen novel analgesics, we expected that the CPP paradigm could also be used to screen novel analgesics in the paclitaxel model of CIPN. Specifically, we
predicted that treatment with MJN110 would produce a CPP for the MJN110-associated chamber in paclitaxel-treated, but not control vehicle-treated, mice. In paclitaxel-treated mice, MJN110 administration produced a significant place preference for the MJN110-associated chamber that was not observed in control vehicle-treated mice. This finding that MJN110 produces a CPP in paclitaxel-, but not vehicle-treated mice indicates that MJN110 relieves an aversive state associated with paclitaxel treatment. While this result could indicate relief from the sensory symptoms of CIPN, as interpreted for candidate analgesics in the cisplatin model of CIPN (Park et al., 2013; Krukowski et al., 2017), preference for the MJN110-associated chamber in paclitaxel-treated mice could indicate relief from an aversive state distinct from neuropathy.

Mice treated with paclitaxel demonstrate a preference for the dark chamber of the light/dark box apparatus (Toma et al., 2017), which is used to screen candidate anxiolytic drugs, such as diazepam, that increase time spent in the light chamber (Young and Johnson, 1991). In naïve mice, JZL184 treatment increases preference for the light chamber of the light/dark box apparatus (Bedse et al., 2017). Thus, the CPP produced by MJN110 treatment could also indicate relief from an anxiety-like phenotype or other untoward long-lasting effects from paclitaxel treatment distinct from neuropathy. One way to separate these different possible outcomes would be to test JZL184 and MJN110 in the light/dark box and forced swim test with paclitaxel-treated mice. As JZL184 does not produce a CPP in naïve (Gamage et al., 2015) or vehicle control (Chapter 2) mice, the ability of MAGL inhibition to reverse a paclitaxel-induced preference for the dark chamber of the light/dark box test also needs to be assessed. A pattern of findings in which MAGL inhibition does not increase preference for the light side of the light/dark box or decrease immobility in the forced swim test treatment would suggest that CPP development could be due to antinociceptive effects.
Monoacylglycerol lipase inhibitors reverse markers of paclitaxel-induced inflammation in the lumbar spinal cord and associated DRG

Paclitaxel treatment causes direct neurotoxicity and—depending on the anatomic location—inflammation in peripheral nerves, DRG and the dorsal horn of the spinal cord (Peters et al., 2007). As CB₁ receptors are found on neurons (Devane et al., 1988; Hohmann and Herkenham, 1999) and CB₂ receptors on cells of the immune system (Munro et al., 1993; Romero-Sandoval et al., 2009a), we predicted that MAGL inhibition will reduce paclitaxel-induced markers of inflammation associated with allodynia. Specifically, we examine MCP-1 and phospho-p38 expression in the lumbar DRG and spinal cord of paclitaxel-treated mice following MAGL inhibition.

In the DRG and lumbar dorsal horn, paclitaxel induces expression of MCP-1 in neurons (Zhang et al., 2013, 2016a) which is required for allodynia development and maintenance. In the DRG, MCP-1 expression has two primary effects: (1) autocrine and paracrine receptor-mediated sensitization of sensory afferent neurons (Jung et al., 2008; Zhang et al., 2013) and (2) peripheral macrophage chemotaxis and accumulation in the DRG (Zhang et al., 2016a). In the spinal cord, MCP-1 is expressed by astrocytes; however, the function of MCP-1 in this tissue is unclear as its receptor is not expressed (Zhang et al., 2013). Because the direct CB₂ receptor agonist, AM1710, attenuates spinal MCP-1 expression in paclitaxel-treated mice (Deng et al., 2015b), we predicted that MAGL inhibition would attenuate MCP-1 expression in both the DRG and spinal cord of our mouse model of paclitaxel CIPN. Using immunohistochemical analysis, we demonstrate that MCP-1 expression was increased in the DRG and spinal dorsal horn of paclitaxel-treated mice compared to vehicle control. Treatment with MJN110 at a time point corresponding with allodynia reversal, attenuated MCP-1 expression in both tissues. This finding suggests that
decreased MCP-1 expression is one potential mechanism for allodynia reversal by MAGL inhibition as antagonism of the MCP-1 receptor reverses paclitaxel-induced mechanical allodynia (Al-Mazidi et al., 2017). However, a causal relationship needs to be established. One way to do this would be to administer exogenous MCP-1, by intrathecal injection, into paclitaxel-treated mice following MJN110 treatment. Restoration of allodynia following MCP-1 treatment would suggest that MJN110 causes reversal of mechanical allodynia by decreasing MCP-1 expression. However, as MCP-1 directly sensitizes sensory neurons and MCP-1 antagonists reverse paclitaxel allodynia (Al-Mazidi et al., 2017), MCP-1 administration could induce allodynia. Therefore, this type of experiment would need to be appropriately controlled to ensure that MCP-1 does not induce allodynia in control vehicle-treated mice.

In addition to increasing MCP-1 expression (Zhang et al., 2013), paclitaxel treatment increases the phosphorylation of p38 MAPK in DRG neurons (Li et al., 2015). Phosphorylation (activation) of p38 in neurons is secondary to TLR4 activation (Li et al., 2015) and can cause sensitization through sodium channel phosphorylation (Hudmon et al., 2008) and activation of the NF-κB pathway, which controls the expression of a variety of pro-inflammatory mediators such as TNF-α, IL-1β and IL-6 contributing to nociception (Lin et al., 2014). However, phosphorylation of p38 is not observed in the spinal cord of paclitaxel-treated mice (Li et al., 2015), indicating that this mechanism is limited to the DRG. Because the direct CB2 receptor agonist, AM1710, attenuates DRG phospho-p38 expression the CCI model of neuropathy (Wilkerson et al., 2012a), we predicted that MAGL inhibition would attenuate phospho-p38 expression in the DRG of our mouse model of paclitaxel CIPN. Using immunohistochemical analysis, we found that phospho-p38 expression was significantly increased in the DRG, but not the spinal dorsal horn, of paclitaxel-treated mice compared to vehicle control. Treatment with
MJN110, at a time point corresponding with allodynia reversal, attenuated phospho-p38 expression in DRG without affecting basal phospho-p38 expression in the spinal dorsal horn. While this corresponds with previous similar findings in CCI model of neuropathy using a CB₂ receptor agonist (Wilkerson et al., 2012a), MJN110 treatment could cause CB₁ receptor activation, which activates p38 MAPK (Derkinderen et al., 2001). Therefore, this anti-inflammatory effect of MJN110 may be CB₂-dependent with attenuated expression of other cytokines associated with paclitaxel neuropathy, such as TNF-α (Deng et al., 2015b; Wu et al., 2015), that cause p38 activation (Barbin et al., 2001). Decreased phospho-p38 expression by MJN110 treatment could also be due to decreased MCP-1 release, outlined above, as MCP-1 can activate the p38-MAPK pathway in neurons (Cho and Gruol, 2008). This could be tested via intrathecal administration of anti-MCP-1 antibodies followed by staining for phospho-p38 expression. Co-localization analysis using DRG tissues from paclitaxel- and MJN110-treated mice reveals that MCP-1 and phospho-p38 expression in paclitaxel-treated mice co-localizes to neurons and cells consistent with the localization of satellite cells.

While both MCP-1 (Zhang et al., 2013, 2016a) and phospho-p38 (Li et al., 2015) are critical for paclitaxel-induced mechanical allodynia, MAGL inhibitors may also attenuate other markers of inflammation through both CB₁ and CB₂ receptor activation. In the periphery, paclitaxel causes direct toxicity to sensory afferents due to axonal transport deficits (Bobylev et al., 2015) and spontaneous action potential generation (Xiao and Bennett, 2008). Activation of peripheral CB₁ receptors (Hohmann and Herkenham, 1999) secondary to MAGL inhibition could attenuate hypersensitivity of sensory afferents. CB₁ receptor activation in the DRG (Hohmann and Herkenham, 1999) and spinal dorsal horn (Hohmann et al., 1999) could attenuate hyperexcitability of DRG (Li et al., 2017b) and spinal (Cata et al., 2006) neurons of paclitaxel-
treated mice. CB$_2$ receptor activation can also suppress inflammation associated with paclitaxel allostynia. Macrophage treatment with the CB$_2$ agonist JWH015 suppresses MCP-1-induced chemotaxis (Montecucco et al., 2008). This could prevent macrophage accumulation in the DRG of paclitaxel-treated mice (Zhang et al., 2016a). Similarly, in the spinal cord, CB$_2$ activation by MAGL inhibition could attenuate microglia activation (Romero-Sandoval et al., 2009b) and pro-inflammatory cytokine production (Deng et al., 2015b) (i.e., MCP-1, IL-1β, TNFα) associated with paclitaxel treatment (Peters et al., 2007; Burgos et al., 2012; Pevida et al., 2013; Deng et al., 2015b).

*Monoacylglycerol lipase inhibitors do not affect the chemotherapeutic efficacy of paclitaxel in cell lines of non-small cell lung cancer*

As MAGL inhibition is anti-inflammatory and would be administered during or after paclitaxel chemotherapy, it is important to establish that MAGL inhibitors do not interfere with the anti-tumor effects of paclitaxel in cancer cells. Cell treatment with paclitaxel stabilizes microtubules (Schiff and Horwitz, 1980), initiating cell death by arresting progression through mitosis (De Brabander et al., 1981), which is thought to be the mechanism of paclitaxel chemotherapy. Because MAGL inhibition or knockdown in cancer cells decreases cancer cell migration, invasion and growth by disrupting fatty acid regulation (Nomura et al., 2010; Zhang et al., 2016b), we predicted that MAGL inhibition would not interfere with, but could enhance, the anti-growth or apoptotic effects of paclitaxel in non-small cell lung cancer. Treatment of A549 or H460 with paclitaxel decreased cell viability and increased apoptosis, effects that were not altered by co-treatment with JZL184 at a concentration known to inhibit MAGL in cancer cells (Nomura et al., 2010). This suggests that MAGL inhibition does not interfere with the anti-tumor effects of paclitaxel, at least in non-small cell lung cancer. While it is unknown if these
cancer cells express MAGL, non-small cell lung cancers can express CB₁ or CB₂ receptors (Preet et al., 2011). Treatment of A549 cells with the dual CB₁/CB₂ receptor agonist WIN55,212 or the CB₂ agonist JWH015 significantly reduced cell growth and migration in vitro in a cannabinoid-receptor dependent manner (Preet et al., 2011). Taken together, these results suggest that inhibition of MAGL could enhance the anti-tumor effects of paclitaxel in other cancer types by (1) decreasing fatty acids mediating cancer cell invasion (Nomura et al., 2010) and (2) stimulating cannabinoid receptors (Preet et al., 2011).

**MAGL inhibition prevents the development of paclitaxel-induced mechanical allodynia**

As paclitaxel neuropathies can persist in as many as 41% of patients three years after chemotherapy (Tanabe et al., 2013), prevention of neuropathy development would decrease the need for extensive pharmacotherapy after neuropathy onset. In rodents, co-administration of either the dual cannabinoid receptor agonist WIN55,212 (Burgos et al., 2012; Rahn et al., 2014) or CB₂ agonists (AM1710 (Rahn et al., 2014) or MDA7 (Naguib et al., 2012)) with paclitaxel prevents the development of both mechanical allodynia and makers of CNS inflammation associated with neuropathy development. Based on these previous results, we hypothesized that co-treatment with the MAGL inhibitors JZL184 or MJN110 with paclitaxel would prevent the development of mechanical allodynia in mice. Because of the potential for decreased CB₁ expression and function due to repeated administration of 40 mg/kg JZL184 (Schlosburg et al., 2010; Kinsey et al., 2013), as previously discussed, we also used a low-dose (4 mg/kg) treatment regimen that spares CB₁ expression and retains anti-allodynic effects (Kinsey et al., 2013). Treatment with 40 mg/kg JZL184 during a cycle of paclitaxel prevented the development of mechanical allodynia up to two weeks following paclitaxel cessation. Although treatment with low-dose JZL184, 4 mg/kg, did not prevent allodynia one day after paclitaxel cessation, recovery
from allodynia was enhanced. While paclitaxel-induced mechanical allodynia in C57BL/6J mice can persist for up to three months in our mouse model (Toma et al., 2017), co-treatment with low-dose JZL184 caused recovery of allodynia at two weeks post-paclitaxel. Because JZL184 can also elevate AEA with repeated administration (Kinsey et al., 2013) by inhibiting FAAH (Long et al., 2009a), we also tested MJN110, which does not inhibit FAAH (Niphakis et al., 2013). Co-administration of MJN110 with paclitaxel prevented the development of mechanical allodynia up to one week after paclitaxel cessation, suggesting that MAGL inhibition contributes to the preventative effects observed with JZL184 treatment. Lastly, we tested our hypothesis that prevention of paclitaxel allodynia was CB1-dependent. Co-administration of 40 mg/kg JZL184 with paclitaxel prevented the development of mechanical allodynia in both CB1 (+/+) and (−/−) mice two weeks after paclitaxel cessation. This is consistent with prevention of allodynia with 40 mg/kg JZL184 in C57BL/6J mice as repeated administration of 40 mg/kg JZL184 causes decreased CB1 expression and function (Schlosburg et al., 2010; Kinsey et al., 2013). Because prevention of alldodynia is not CB1-mediated, other actions of MAGL inhibition, such as CB2 receptor activation or decreased arachidonic acid metabolism, may mediate prevention of allodynia demonstrated here. Additionally, elevated AEA with repeated administration of JZL184 may also play a role.

While MJN110 prevented the development of mechanical allodynia one day and one week post-paclitaxel, allodynia was not prevented compared to paclitaxel control at two weeks. Though, MJN110-treated mice did not differ from vehicle control mice at this time point suggesting a possible lack of power here. These data suggest that prevention of allodynia by inhibition of MAGL produces a transient or partial protective effect. As co-treatment with 40 mg/kg JZL184 prevented allodynia at two weeks post-paclitaxel, prevention of alldodynia may be
secondary to increased AEA in the brain (Kinsey et al., 2013) and spinal cord (Chapter 2). One way to test this possibility is by co-administration a selective FAAH inhibitor, such as PF-3845 (Ahn et al., 2009), with paclitaxel. Prevention of allodynia with a FAAH inhibitor, especially at the two-week time point, would suggest that FAAH inhibition with repeated JZL184 administration could play a role in the preventative effect here. However the mechanism of AEA-mediated protection could occur outside of the spinal cord or brain as FAAH inhibition outside of these tissues reverses CCI-induced mechanical allodynia (Clapper et al., 2010). It is also possible that other lipids metabolized by FAAH exert a protective effect at the two-week timepoint. Palmitoylethanolamide (PEA) is also metabolized by FAAH (Tiger et al., 2000) and reverses paclitaxel-induced allodynia through a cannabinoid receptor-independent mechanism (Donvito et al., 2016). PEA is also elevated in the brain with repeated administration of JZL184 (Kinsey et al., 2013). Co-administration of PEA with the oxaliplatin chemotherapy prevents mechanical allodynia, alterations in DRG neuronal morphology and partially attenuates spinal neuronal hyperexcitability (Di Cesare Mannelli et al., 2015).

There are multiple potential mechanisms of allodynia prevention by MAGL inhibition in both the peripheral and central nervous system. These mechanisms overlap substantially with the mechanisms of allodynia reversal, discussed above, with the prevention of paclitaxel-induced neuronal hypersensitivity and inflammation. Because the CB1 receptor is not required for allodynia prevention with MAGL inhibition, we predict that the anti-inflammatory effects of CB2 receptor activation mediate the preventative effects shown here. Prevention of allodynia could also be cannabinoid receptor-independent, as discussed below. Co-administration of CB2-selective agonists, AM1710 (Rahn et al., 2014) and MDA7 (Naguib et al., 2012), prevent the development of paclitaxel-induced mechanical alldodynia and, for MDA7, prevent paclitaxel-
induced microglia (CD11b expression) and astrocyte (GFAP expression) activation in the spinal dorsal horn associated with allodynia development (Naguib et al., 2012). Thus, prevention of microglia-associated inflammation (Pevida et al., 2013) or astrocyte-induced excitatory glutamate accumulation (Zhang et al., 2012) in the spinal dorsal horn could prevent the development of mechanical allodynia. Other possibilities include prevention of MCP-1 expression (Zhang et al., 2013) or phosphorylation of p38 (Li et al., 2015) in the DRG, which are necessary for allodynia development. Activation of the CB2 receptor cold also depresses macrophage chemotaxis (Montecucco et al., 2008), preventing accumulation in the DRG following paclitaxel treatment (Zhang et al., 2016a), and preventing neuropathy development.

In addition to a CB2-mediated mechanism, protection from allodynia could also involve a reduction in arachidonic acid levels and downstream pro-inflammatory mediators. In the brain, inhibition of MAGL following LPS administration reduces arachidonic acid synthesis, as well as the expression of pro-inflammatory arachidonic acid metabolites, such as PGE2 and PGD2, as well as cytokines such as IL-1β and TNFα (Nomura et al., 2011b). In the spinal cord, repeated administration of 40 mg/kg JZL184 significantly reduced arachidonic acid levels in paclitaxel-treated mice (Chapter 2). Therefore, reduced expression of arachidonic acid, pro-inflammatory metabolites, and cytokine levels in the spinal cord could prevent the development of central sensitization required for allodynia. This reduction of inflammation could also occur in the DRG. Lastly, control of fatty acid metabolism by MAGL could also play a role. Inhibition of MAGL decreases LPA levels in cancer cells (Nomura et al., 2010), and mice lacking LPA receptor expression fail to develop paclitaxel-induced mechanical allodynia (Uchida et al., 2014). Therefore, decreased LPA levels could contribute to the preventative effect shown here. However, it is unknown if MAGL regulates LPA levels in neuronal tissues.
**Overall Conclusions**

In this dissertation, we establish MAGL inhibition as a potential therapeutic strategy for both the treatment and prevention of paclitaxel-induced mechanical allodynia in a mouse model. After allodynia onset, inhibition of MAGL with either JZL184 or MJN110 reverses mechanical allodynia in time- and dose-dependent manners. These anti-allodynic effects require both CB$_1$ and CB$_2$ receptor expression and function as demonstrated using genetic and pharmacologic approaches. As the anti-allodynic effects of 40 mg/kg JZL184 are not maintained with repeated administration, we establish that repeated administration of 4 mg/kg JZL184 increases paw withdrawal thresholds to levels comparable with control vehicle-treated mice. MAGL inhibition also reverses an affective state in paclitaxel-treated, but not control vehicle-treated, mice as demonstrated by a place preference for the MJN110-associated chamber in the CPP paradigm. As a possible mechanism for allodynia reversal, we establish that MJN110 treatment attenuates markers of inflammation associated with paclitaxel allodynia, namely MCP-1 and phospho-p38 in the DRG as well as MCP-1 in the spinal dorsal horn. We also show that the anti-proliferative and apoptotic effects of paclitaxel on non-small cell lung cancer cells are maintained with JZL184 treatment. Finally, we demonstrate that co-administration of MAGL inhibitors with paclitaxel prevents the development of mechanical allodynia through a mechanism that is CB$_1$ receptor-independent.

Taken together, these studies indicate that MAGL is a promising target for both the reversal and prevention of paclitaxel CIPN in the clinic. Our results indicate that MAGL inhibition could improve both the sensory (Dougherty et al., 2004) and affective/motivational (Hong et al., 2014; Zanville et al., 2016) impacts of CIPN by attenuating neuropathy onset and related inflammation. Importantly, MAGL inhibition is likely to be safe during chemotherapy.
treatment as MAGL inhibition does not interfere with the anti-proliferative or anti-apoptotic effects of paclitaxel in cell lines of non-small cell lung cancer. Finally, our results suggest that MAGL inhibition can prevent—or at least shorten the duration of—paclitaxel CIPN. This would be of great clinical benefit as paclitaxel CIPN can persist for years after chemotherapy (Tanabe et al., 2013). Given these promising preclinical results for paclitaxel CIPN, along with potential benefits for other types of CIPN (Guindon et al., 2013) and neuropathic pain (Ignatowska-Jankowska et al., 2015), MAGL inhibitors warrant further development as a novel class of analgesics.

**Future Directions**

Given the need for novel analgesics to both treat and prevent paclitaxel CIPN, further research is needed to elucidate both mechanisms of neuropathy development and the impact of MAGL inhibition in mouse models. While we have shown that reversal of allodynia by both JZL184 and MJN110 requires both CB₁ and CB₂ receptors using complementary genetic and pharmacologic approaches, the mechanisms contributing to allodynia development and maintenance in CB₁ and CB₂ knockout mice needs to be elucidated. This is especially important regarding expression of phospho-p38 MAPK and MCP-1. By extending the immunohistochemical experiment used in Chapter 2 to include CB₁ and CB₂ (-/-) mice and (+/+), reduction in phospho-p38 or MCP-1 expression can be linked to one or the other cannabinoid receptor. This could suggest whether reduced expression of MCP-1 and/or phospho-p38 by MAGL inhibition is CB₁- or CB₂-mediated. This experiment is also needed as the mechanisms of alldynia development in the spinal cord and DRG could differ between CB₁ or CB₂ (-/-) mice and (+/+). Thus, the lack of anti-allodynic effects from either MJN110 or JZL184 in (-/-) mice could be due to alternative mechanisms of alldynia development, such as
enhanced neuronal hypersensitivity in CB<sub>1</sub> (-/-) mice or microglia activation in CB<sub>2</sub> (-/-) mice. Lastly, the experiments using semi-quantitative immunohistochemistry methods here need to be replicated using more quantitative techniques: western blotting for protein expression and/or enzyme-linked immunosorbent assays for cytokine expression.

One way to further dissociate the CB<sub>1</sub> versus CB<sub>2</sub> receptor-mediated contributions to the anti-allodynic effects of MAGL inhibitors would be to use of CB<sub>1</sub> or CB<sub>2</sub> receptor-floxed mice. These mice would allow for dissociation of cell-type specific effects. In particular, decreased CB<sub>1</sub> receptor expression in primary sensory afferents could be used to distinguish between the contributions of primary and secondary sensory afferents to the anti-allodynic effects of MAGL inhibitors. Because paclitaxel has direct neurotoxic and pro-inflammatory effects in the DRG and spinal dorsal horn impacting primary sensory afferents (Zhang et al., 2013; Li et al., 2017b), we predict MAGL inhibitors will not reverse allodynia that mice lacking CB<sub>1</sub> receptor expression primary sensory afferents. Descending inhibition from supraspinal neurons expressing the CB<sub>1</sub> receptor may also be required for the anti-allodynic effects of MAGL inhibitors. Decreased CB<sub>2</sub> receptor expression in specific cell types could also be used to determine the contribution of immune system cells, especially macrophages, to the anti-allodynic effects of MAGL inhibitors. These behavioral studies could also be extended in female mice to examine for potential sex-specific differences in neuropathy development and antinociceptive effects. Given that macrophage accumulation in the DRG is required for paclitaxel allodynia (Zhang et al., 2016a) and that MCP-1-mediated chemotaxis can be suppressed by a CB<sub>2</sub> receptor agonist, JWH015 (Montecucco et al., 2008), we predict that MAGL inhibition will not reverse mechanical allodynia in mice lacking CB<sub>2</sub> expression on macrophages. This approach could also be used to examine the CB<sub>2</sub> receptor on microglia in the dorsal horn, which may contribute to paclitaxel
allodynia (Pevida et al., 2013), and the anti-allodynic effects of MAGL inhibitors.

While repeated administration of 4 mg/kg JZL184 led to the development of anti-allodynic effects compared to tolerance to the anti-allodynic effects of repeated administration of 40 mg/kg JZL184, this experiment needs to be replicated using MJN110. We hypothesize that repeated administration of high-dose (i.e., 5 mg/kg) MJN110 for six days will cause the development of tolerance to the anti-allodynic effects while repeated administration of a low-dose (i.e., 0.3 mg/kg) will produce anti-allodynic effects. Furthermore, we predict that CB1 receptor expression and function will be decreased in the spinal cord following repeated administration of high-dose MJN110 or JZL184, as seen in the whole brain of mice following CCI surgery (Schlosburg et al., 2010; Kinsey et al., 2013). By using CB1 receptor floxed mice lacking CB1 receptor expression in the brain or spinal cord (i.e., on primary sensory afferents), the location of CB1 receptors required for anti-allodynic effects could be inferred. As paclitaxel CIPN can persist for years in patients following chemotherapy (Tanabe et al., 2013), the length of the repeated administration study needs to be increased. If the duration of repeated low-dose JZL184 treatment is extended beyond six days, 2-AG levels in the CNS may increase to that extent that tolerance develops to the anti-allodynic effects through downregulation of the CB1 receptor. However, it is also possible that a steady state may develop, in which synthesis and degradation of 2-AG are balanced.

As the administration of MJN110 produced a conditioned place preference in paclitaxel-treated mice that was not observed in control vehicle-treated mice, several further experiments are needed. It is important to establish that the development of a CPP is due to antinociceptive effects versus relief of another affective state associated with paclitaxel administration. One way to do this would be to test if MAGL inhibitors attenuate paclitaxel-induced changes in light/dark
box and forced swim test behaviors. The CPP paradigm could also be used to establish whether the anti-nociceptive effects of MJN110 require both the CB₁ and CB₂ receptors. Furthermore, alternative methods of assessing nociception and analgesia in rodent models, such as pain-depressed behaviors, need to be incorporated here. For example, gait analysis of paclitaxel-treated mice demonstrates decreased hind paw contact with the flooring during locomotion (Huehnchen et al., 2013). While mechanical allodynia is reversed by gabapentin administration, gait alterations are not corrected, suggesting that gabapentin may not be antinociceptive. Similarly, in humans, gabapentin is not recommended for the treatment of paclitaxel CIPN (Kim et al., 2015; Loprinzi, 2017), but it is used to treat other types of neuropathic pain (Finnerup et al., 2015). Therefore, this method may be used to assess the antinociceptive effects of MAGL inhibition.

In the A549 and H460 lines of non-small cell lung cancer, co-treatment of JZL184 with paclitaxel did not interfere with the anti-proliferative or anti-apoptotic effects of paclitaxel (Chapter 2). While the dose of JZL184 used here is known to inhibit MAGL in vitro (Nomura et al., 2010), it is unknown whether A549 or H460 cell express MAGL. Expression of MAGL in these cells needs to be confirmed and this experiment needs to be extended to other cancer cell types as paclitaxel is used to treat a wide variety of cancers (WHO, 2017). In particular, MAGL inhibitors need to be assessed in cancer cell lines known to express MAGL to increase cellular free fatty acids associated with an aggressive phenotype (Nomura et al., 2010). The combined use of MAGL with paclitaxel also needs to be used to assess tumor viability in vivo with implantation of human cancer cells in nude mice. Though nude mice would lack proper host immune responses in the tumor microenvironment (Gajewski et al., 2013). These additional experiments would suggest whether MAGL inhibition interferes with—or even potentially
enhances—the anti-tumor effects of paclitaxel in a wider variety of tumors to establish safety for combined use.

In addition to our studies investigating reversal of paclitaxel-induced allodynia after onset, we demonstrate that co-administration of JZL184 or MJN110 with paclitaxel prevents allodynia development. As we demonstrate that co-administration of 40 mg/kg JZL184 prevents allodynia development up to two weeks after paclitaxel cessation, future studies need to examine the duration of allodynia prevention here. Next, the ABHD6 inhibitor KT195 needs to be assessed for prevention of allodynia to control for this off-target effect of MJN110. However, given prevention with 40 mg/kg JZL184 and the failure of KT195 to reverse paclitaxel allodynia after onset, we predict that co-administration of KT195 with paclitaxel will not prevent allodynia development. Lastly, as CB1 (-/-) mice demonstrate prevention of allodynia with 40 mg/kg JZL184, we predict that prevention of allodynia is CB2-mediated. Therefore, prevention of allodynia with JZL184 needs to be tested in both CB2 (-/-) and (+/+). Prevention of allodynia development in CB2 (+/+), but not in (-/-), mice would suggest that the CB2 receptor is required for allodynia prevention with MAGL inhibition. However, prevention of allodynia in both (-/-) and (+/+) mice would suggest that the preventative effect of JZL184 administration occurs in a cannabinoid receptor-independent manner. This could be due to a decrease in arachidonic acid levels in the spinal cord (Chapter 2) and brain (Nomura et al., 2011b; Kinsey et al., 2013), but could also be due to other metabolites regulated by MAGL such as free fatty acids or LPA (Nomura et al., 2010). Another approach would be to co-administer CB1 or CB2 pharmacologic antagonists, such as rimonabant and SR144528, with JZL184. Prevention of allodynia by JZL184 with rimonabant co-treatment, but not SR144528 co-treatment, would suggest that the CB2 receptor is required for allodynia prevention. Because prevention of
allodynia with MJN110 only lasted up to one week post-paclitaxel, the contributions of other lipids, such as AEA and palmitoylethanolamide elevated with repeated JZL184 treatment (Kinsey et al., 2013) needs to be assessed. As previously discussed, elevated AEA or PEA levels could contribute to the protective effects of 40 mg/kg JZL184 two weeks after paclitaxel cessation.

In addition to further behavioral assessment of alldynia prevention, prevention of alldynia needs to be correlated with potential mechanisms of neuropathy. In the spinal cord or DRG, co-administration of a MAGL inhibitor may prevent neuronal hypersensitivity (Cata et al., 2006; Matsumoto et al., 2006) or increased expression of makers of inflammation, such as MCP-1 (Zhang et al., 2013) or phospho-p38 (Li et al., 2015), associated with alldynia development.

Lastly, protection from the peripheral neurotoxic effects of paclitaxel also needs to be established. Intraepidermal nerve fiber loss occurs as a secondary consequence of disrupted neuronal homeostasis preventing peripheral regeneration with keratinocyte turnover (Gornstein and Schwarz, 2017). As demonstrated by other agents preventing paclitaxel alldynia, such as minocycline (Liu et al., 2010) or nicotine (Kyte et al., 2017), alldynia prevention can be linked with prevention of intraepidermal nerve fiber loss in the hind paw skin. Because MAGL inhibition could dampen both neuronal hypersensitivity and inflammation associated with paclitaxel, we hypothesize that MAGL inhibitor co-administration with paclitaxel will prevent this loss of intraepidermal nerve fibers.

CIPN is a long-lasting side-effect of paclitaxel chemotherapy causing serious sensory impairments (Dougherty et al., 2004; Tanabe et al., 2013) without effective treatment options (Loprinzi, 2017). As paclitaxel is used throughout the world to treat various cancers (WHO, 2017), there is a serious need to identify novel analgesic strategies capable of both treating CIPN
after onset and preventing CIPN development. In this dissertation, we establish MAGL as a novel target for the reversal of paclitaxel-induced allodynia and inflammation in a mouse model. We also demonstrate treatment of a paclitaxel-induced affective state in mice using the conditioned place preference paradigm and show that MAGL inhibition does not interfere with the anti-tumor effect of paclitaxel in vitro. Lastly, we demonstrate that MAGL inhibition is capable of preventing allodynia development prior to onset. The results presented here suggest that MAGL inhibitors may be useful for the clinical management of paclitaxel CIPN. As efficacious treatment strategies are strongly needed for the management of paclitaxel CIPN, MAGL inhibitors deserve further clinical consideration.
List of References


Li, Y., Zhang, H., Zhang, H., Kosturakis, A.K., Jawad, A.B., and Dougherty, P.M. (2014). Toll-


Pfizer (2008). Prevention And Treatment Of Chemotherapy-Induced Peripheral Neuropathy In Subjects With Advanced Colorectal Cancer.


binding and mRNA levels in several rat brain regions. Brain Res. Mol. Brain Res. 46: 100–108.


Structural basis for induction of peripheral neuropathy by microtubule-targeting cancer drugs.
Cancer Res. 76: 5115–5123.


Sugiura, T., Kodaka, T., Kondo, S., Tonegawa, T., Nakane, S., Kishimoto, S., et al. (1996). 2-


Thompson, W.L., and Eldik, L.J. Van (2009). Inflammatory cytokines stimulate the chemokines
CCL2/MCP-1 and CCL7/MCP-7 through NFκB and MAPK dependent pathways in rat astrocytes. Brain Res. 1287: 47–57.


Induction of monocyte chemoattractant protein-1 (mcp-1) and its receptor ccr2 in primary sensory neurons contributes to paclitaxel-induced peripheral neuropathy. J. Pain 14: 1031–1044.


Vita

Zachary Adam Curry was born on August 8, 1989 in Falls Church, Virginia. He is an American citizen. In 2012, he graduated summa cum laude with an Honors Bachelor of Science from Winthrop University in Rock Hill, South Carolina. There he majored in Chemistry with a concertation in Biochemistry with minors in Biology and Mathematics. During his undergraduate education he conducted research at St. Jude Children's Research Hospital in Memphis, Tennessee under the mentorship of Jie Zheng, Ph.D. in the Department of Structural Biology. There he was a member of the 2010 and 2011 Pediatric Oncology Education Programs. In 2012 he joined the M.D./Ph.D. program at Virginia Commonwealth University in Richmond, Virginia where he completed his Ph.D. studies investigating the impact of monoacylglycerol lipase inhibition on paclitaxel-induced mechanical allodynia in mice under the mentorship of Aron Lichtman, Ph.D. He has presented his work at several conferences including the International Cannabinoid Research Society Symposium on the Cannabinoids (2017, Montréal, Canada) where he received a travel award for oral presentation and the National MD/PhD Student Conference (2016, Keystone, Colorado) where he received a diversity travel award.

Publications:

