

THE ROLES OF THE ENDOGENOUS CANNABINOID SYSTEM IN CANCER-  
INDUCED BONE PAIN AND OPIOID-INDUCED REWARD

by

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A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

WITH A MAJOR IN MEDICAL PHARMACOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2020

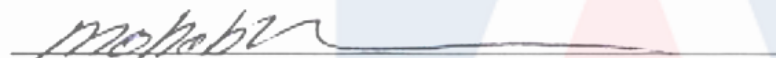
THE UNIVERSITY OF ARIZONA  
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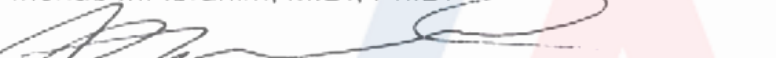
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## ACKNOWLEDGEMENTS

“Time is like a running horse, who swiftly passes through a crevice.” This is a Chinese proverb describing how time flies so quickly in our life. Now, it is exactly described how I feel about my 5-year Ph.D. training. When looking back to this long journey, I am very pleased that I have achieved my initial goal when I just attended graduate school – becoming a qualified researcher who is able to make contribution to the scientific world. Undoubtedly, my goal would not have been possible without the support of many people.

I am immensely grateful to my advisor Dr. Todd Vanderah for all the invaluable guidance and suggestions he provided to me for both my research and my life. His words have always inspired me and pushed me forward every time I thought I have reached my limits. I will always keep these words in my mind and try my best to be a greater scientist and a better person during my lifetime.

I am truly thankful to my committee members Drs. Tally Largent-Milnes, John Streicher, Edward French, and Mohab Ibrahim. I deeply appreciate their selfless help on my experiments especially when I encountered difficulties, as well as their sincere encouragement when I made achievements.

My deep appreciation also goes to all of the previous and current students and co-workers in the Vanderah and Largent-Milnes labs as well as the Department of Pharmacology - Dr. Lauren Slosky, Dr. Alexander Sandweiss, Dr. Shaness Grenald, Dr. Karissa Cottier, Dr. William Staatz, Austen Thompson, Austin Flohrschutz, Maha Sulaiman, Alysha Swanson, Kelly Karlage, Dr. Erika Liktov-Busa, Jared Wahl, Seph Palomino, Beth Wiese, Vani Verkhovsky, Dominique Lund, Haley Ciccone, Holly Ellingson, Dez Coleman, Ryan Vanderah, Angela Smith, Dr. Frank Porreca, Dr. Lusine Gomtsian, Nathan Eyde, Donna Lu, Dr. Wei Lei, Dr. Attila Keresztes, Justin Lavigne, Dr. Aubin Moutal, Dr. Razaz Felemban, and Dr. Wazir Abdullahi. Thank you for being with me all the way. It is your generous help and support that made this challenging journey a pleasant and fruitful one. I would like to specifically thank Dr. Lusine Gomtsian, an extremely kind and considerate colleague and friend, who provided the best possible help for me a foreigner to settle down and get used to the life in U.S.

Lastly, I would like to acknowledge with gratitude to my family – my parents, Gaoyi Zhang and Lingli Yang, as well as my grandma Yuanxiu Tang, who constantly provide their support and love throughout my pursuit of this degree; to my girlfriend, Yanxia Chen, who always encourages me when I experienced failures and applauded for me when I made great progresses; to all the Chinese friends I have met at the University of Arizona – Dr. Xuezheng Ge, Dr. Kun Xiong, Dr. Mengdie Wang, Dr. Amy Wong, Xinyuan Liu and Nan Song, who bring me a feeling of home at this different side of the world.

This work was made possible with the financial support from the NIH National Cancer Institute grant R01CA142115 (Dr. Todd Vanderah) and UC Center for Accelerated Innovation grant U54HL119893 (Dr. Igor Spigelman).

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## **ABSTRACT**

Chronic pain is a severe problem affecting over 20% of adults worldwide. Opioids, as the current most effective analgesics, are commonly prescribed to manage this devastating symptom. However, the use of opioids is frequently associated with serious side effects, such as respiratory depression and bone degradation. In addition, as opioids possess a profound addiction potential, the wide prescription of opioids has led to a severe opioid epidemic in North America, particularly in the United States. Recently, the endogenous cannabinoid system has emerged as a promising therapeutic target for the treatment of chronic pain and opioid addiction. However, there are still many questions in need of answers before we can translate current research into clinical application. In the present study, we sought to answer two major questions: (1) How can we develop a novel cannabinoid that produces a strong analgesic effect but has limited central side effects? (2) How the endocannabinoid 2-AG and cannabinoid receptor 2 (CB2R) are involved in the chronic opioid-induced reward? Our studies have found that targeting the peripheral CB1Rs can effectively attenuate pain behaviors in a syngeneic murine model of cancer-induced bone pain and results in limited central side effects, as well as other cannabinoid-associated side effects. Furthermore, the elevation of 2-AG tone and activation of CB2Rs remarkably inhibits chronic morphine-induced reward behavior. Yet, chronic morphine exposure reduces the expression of CB2Rs but does not significantly change the production of 2-AG in the VTA. These data suggest that (1) targeting peripheral CB1Rs can be a valuable

therapeutic strategy for treating cancer-induced bone pain, and (2) promoting 2-AG and CB2R signaling may provide therapeutic opportunity for opioid addiction.

## LIST OF ABBREVIATIONS

2-AG	2-Arachidonoylglycerol
ABHD12	Abhydrolase domain containing 12
ABHD6	Abhydrolase domain containing 6
AEA	Anandamide
ASIC3	Acid sensing ion channel subunit 3
CB1R	Cannabinoid receptor 1
CB2R	Cannabinoid receptor 2
CIBP	Cancer-induced bone pain
CNS	Central nervous system
CPP	Conditioned place preference
CRISPR	Clustered regularly interspaced short palindromic repeats
CTX	C-terminal telopeptides of type I collagen
CXCR1	Chemokine (C-X-C motif) receptor 1
DAG	1,2-diacylglycerol
DEXA	Dual-energy x-ray absorptiometry
ERK	Extracellular signal-regulated kinase
FAAH	Fatty acid amide hydrolyase
GABA	Gamma aminobutyric acid
GI	Gastrointestinal
GPCR	G protein-coupled receptor
GPR55	G protein-coupled receptor 55

IL-1 $\beta$	Interleukin-1 $\beta$
LV	Lentivirus
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
mGluR I	Metabotropic glutamate receptor type I
MIP-1A	Macrophage inflammatory protein-1 $\alpha$
MOR	$\mu$ opioid receptor
NAc	Nucleus accumbens
NADA	N-arachidonoyl-dopamine
NAPE	N-arachidonoyl-phosphatidylethanolamine
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAM	Positive allosteric modulator
PCR	Polymerase chain reaction
PI	Phosphatidylinositol
PKC	Protein kinase C
PLA1	Phospholipase A1
PLC	Phospholipase C
PLD	Phospholipase D
PPAR	Peroxisome proliferator-activated receptor

PrNMI	4-{2-[-(1E)-1[(4-propylnaphthalen-1-yl)methylidene]-1H-inden-3-yl]ethyl}morpholine
PTHrP	Parathyroid hormone related protein
qRT-PCR	Quantitative real-time polymerase chain reaction
RANK	Receptor activator of nuclear factor kappa-B
RANK-L	Receptor activator of nuclear factor kappa-B ligand
RMTg	Rostromedial tegmental nucleus
TIDE	Tracking of indels by decomposition
TNF $\alpha$	Tumor necrosis factor $\alpha$
TNFR	Tumor necrosis factor receptor
TrkA	Tropomyosin receptor kinase A
TRPV1	Transient receptor potential cation channel subfamily V member 1
VGCC	Voltage-gated calcium channel
VTA	Ventral tegmental area

# CHAPTER 1: INTRODUCTION

## 1.1 Chronic pain

According to the definition by the International Association for the Study of Pain, pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage [1]. From an evolutionary perspective, the development of this machinery is intended to help us recognize and avoid the actual or potential detrimental factors, thus enhancing the probability of our survival [2]. However, due to a variety of reasons including but not limiting to the persistence of tissue damage, malfunction of pain sensation, transmission and modulation, pain can persist for a long time. The pain that lasts over three months is defined as chronic pain [3].

Chronic pain is an enormous problem globally as it is estimated that over 20% of adults are suffering from this type of pain worldwide with another 10% of adults being newly diagnosed each year [4]. In the United States, approximate 30% of the population is suffering from chronic pain, and a higher prevalence (~40%) is presented among the elder people (65+ years old) [5]. Chronic pain not only produces disabling effects on patients' daily physical activities, but also has severe impacts on their mood and mental health (e.g. increased rates of major depressive disorder, suicidal ideation and attempts), cognitive processes (e.g. memory interruption), and cardiovascular health (e.g. increased risk of hypertension) [6]. Moreover, studies have shown that chronic pain can significantly affect patients' family and social relationships, which largely compromises the quality of patients'

life [7,8]. Based on the initial causes, chronic pain can be classified into chronic cancer pain and chronic non-cancer pain.

## **1.2 Cancer-induced bone pain**

Chronic cancer pain, as a subclass of chronic pain, is a symptom that is frequently reported by the patients who are diagnosed with solid tumors. According to previous study, the overall prevalence of this pain ranges from 15 to 75% depending on the types of cancer, extent of disease progression and other factors [9]. As the detection and treatment of cancer have been improving and resulting in a longer survival time for cancer patients, chronic cancer pain will be a growing problem and affect more patients [10,11]. In cancer patients especially those with malignant tumors, cancer pain is commonly displayed as bone pain since bone is a congenial soil for cancer cell metastasis [12,13]. This pain is frequently described as a mixture of constant background pain and can be exacerbated by movement and weight bearing [14]. The mechanisms of cancer-induced bone pain are not fully understood yet, but current studies indicated that it exhibits the elements of both neuropathic and inflammatory pain as well as bone-specific mechanisms [14].

### **1.2.1 Neuropathic mechanisms of cancer-induced bone pain**

Like skin and other parts of our body, bones are also innervated by primary sensory afferents, particularly unmyelinated C fibers and thinly myelinated A- $\delta$  fibers, and they are primarily concentrated in the periosteum [15]. When metastasizing to the bone, the rapidly proliferating tumor cells can infiltrate and

compress the distal processes of sensory neurons nearby, leading to a morphological change and damage to the neurons and subsequently increase in pain (Figure 1 A) [16]. In addition, previous studies also observed an aberrant sprouting of sensory nerve fibers following the cancer-induced nerve injury in several animal models of bone cancer pain (Figure 1 A) [17–19]. Interestingly, this outgrowth of nerve fibers does not seem to be mutually exclusive to nerve degeneration by tumors, suggesting the nerve injury and regeneration occurs repeatedly in the cancer-bearing bones and may contribute to the worsening pain state [17–19].

Nerve growth factor (NGF) was identified as a key regulator in the abnormal neuronal sprouting in cancer-bearing bones (Figure 1 A). Previous studies found that the majority of the sensory neurons in the periosteum of the bone express tropomyosin receptor kinase A (trkA) receptor, which is the receptor for NGF [18,20,21]. Administration of an anti-NGF antibody significantly inhibited the pathological sprouting of nerve fibers and the formation of neuroma-like structures in two cancer-induced bone pain models [18,19]. More importantly, the cancer-induced pain behaviors were also attenuated in these models [18,19].

In addition to peripheral pain, central mechanisms are also involved in the generation and maintenance of bone cancer pain. This was first revealed by the neurochemical changes in the central nervous system during cancer-induced bone pain. In the spinal cord ipsilateral to the cancer-bearing bone, astrocyte hypertrophy, dynorphin and c-Fos protein expression, and internalization of substance P receptor were observed [22]. Alterations were also observed at the

NMDA receptors. In cancer-bearing mice, increased phosphorylation of a NMDA receptor NR1 and enhanced expression of another NMDA receptor subunit NR2B were observed in the spinal cord of cancer-bearing animals [23,24]. The alterations of the NMDA receptor subunits were accompanied with increased pain behaviors while the blockade of NR1 phosphorylation or NR2B function significantly suppressed cancer-induced bone pain [23,24]. Other studies suggesting the involvement of central mechanisms in bone cancer pain include the identification of unique plastic changes in spinal synaptic transmission mediated by A- $\delta$  and C primary afferents, the proportion shifts of wide dynamic range to nociceptive specific neurons in the superficial dorsal horn, and the significant changes in the descending pain pathway mediated by brainstem [25,26]. Together, these studies have demonstrated the involvement of central sensitization in the development of cancer-induced bone pain.

### **1.2.2 Inflammatory mechanisms of cancer-induced bone pain**

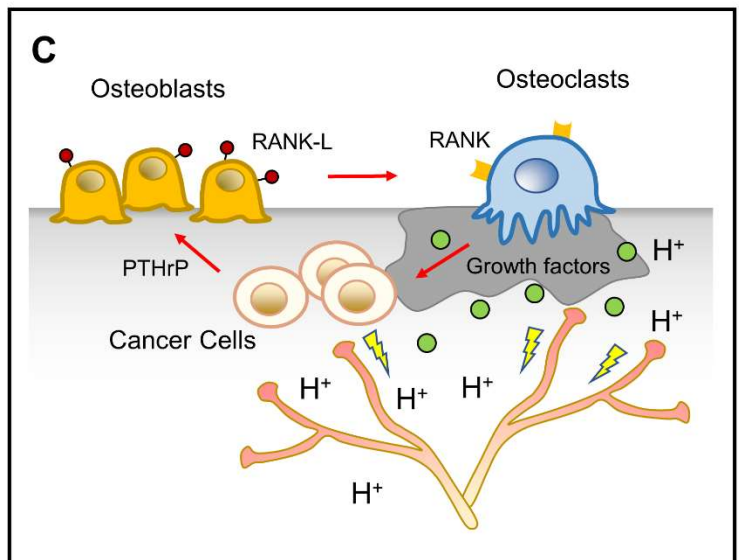
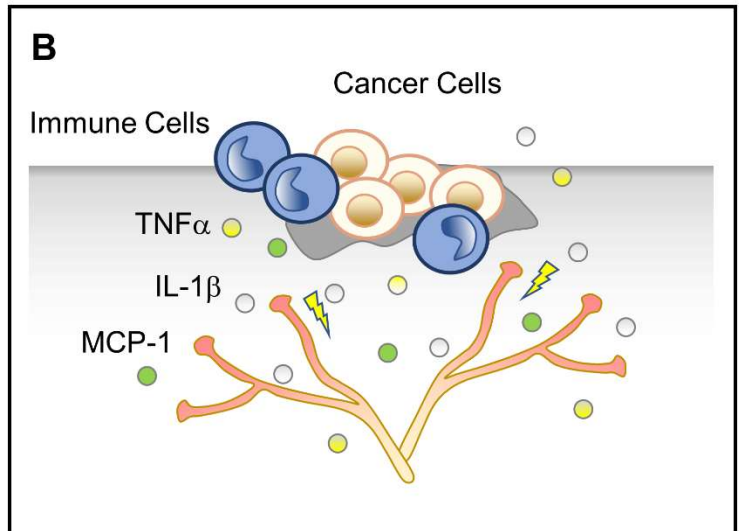
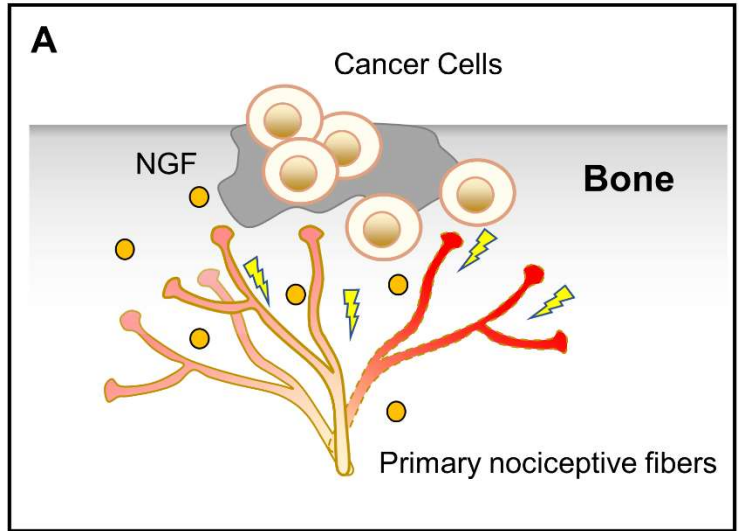
The local inflammation occurring at the tumor-invading sites is also a reason of cancer-induced bone pain. This is usually a result of direct tissue damage caused by cancer growth and the release of pro-inflammatory mediators from cancer cells and the nearby stromal cells (Figure 1 B) [14]. Pro-inflammatory cytokines, the small proteins generally produced by immune cells, were found to implicate in cancer-induced bone pain. IL-1 $\beta$  secreted from cancer cells and spinal cord was found to increase in cancer-bearing animals, and the administration of an IL-1 receptor antagonist can markedly decrease both mechanical and thermal

hypersensitivity [27].  $\text{TNF}\alpha$  is another pro-inflammatory cytokine that is highly expressed in the spinal cord of cancer-bearing animals [28]. Pharmacological blockade of  $\text{TNF}\alpha$  signaling or genetic ablation of its corresponding receptors TNFR1 and TNFR2 can significantly inhibit cancer-induced pain behaviors, spinal astrogliosis and tumor growth, suggesting a pivotal role of  $\text{TNF}\alpha$  signaling in the development of bone cancer pain [28,29]. This regulatory effect of  $\text{TNF}\alpha$  on bone cancer pain is possibly mediated by the sensitization of TRPV1 channels in dorsal root ganglia via the p38/MAP kinase and PKC-dependent pathway [30].

In addition to cytokines, chemokines also play an essential role in cancer-induced bone pain (Figure 1 B). A recent study from our laboratory revealed an up-regulation of two chemokines MCP-1 and MIP-1A in the tumor-burdened femurs in a model of breast cancer-induced bone pain [31]. The expression of these chemokines was significantly reduced after administration of an anti-MCP-1 neutralizing antibody or a cannabinoid receptor 2 agonist, which has been demonstrated to have anti-inflammatory effects previously [31]. Similarly, CXCR1 a chemokine receptor, was found to be up-regulated in the spinal cord of a carcinoma-bearing rats [32,33]. The inhibition of this receptor with a neutralizing antibody decreased mechanical allodynia in the cancer-burdened rats [32,33]. Further studies suggest the involvement of microglial activation and p38-MAPK activation in the CX3CR1-mediated mechanism [32].

### **1.2.3 Bone-specific mechanisms of cancer-induced bone pain**

The skeletons in human and other vertebrates undergoes a constant process of remodeling. The bone-forming cells, osteoblasts and the bone-resorbing cells, osteoclasts are the key players regulating the homeostasis of bone remodeling [34]. In a healthy adult, the bone remodeling is maintained in a steady state. However, when tumor cells metastasize to bone, this balance is disrupted and the bone destruction commonly occurs resulting in mechanical instability, fracture of bone and significant pain (Figure 1 C) [13,15]. The mechanism underlying tumor induces bone degradation involves a “vicious cycle” in bone remodeling (Figure 1 C) [13]. Metastatic cancer cells are capable of producing parathyroid hormone related protein (PTHrP), which induces the expression of RANK-L on the surface of osteoblasts. This increased RANK-L bind to and activate its receptor RANK on the osteoclast precursors, facilitating osteoclastogenesis and bone degradation. Growth factors stored in the bone, including insulin growth factor-1 and tumor growth factor- $\beta$ , are then released and promote cancer cells to generate additional PTHrP, which, in turn, results in a greater bone degradation and worsening pain. In addition to bone instability, bone pain is also caused by the highly acidic environment during bone resorption, which stimulates ASIC3 or TRPV1 channels and produces cancer-induced bone pain (Figure 1 C) [15].



## **Figure 1 Mechanisms of cancer-induced bone pain**

(A) Neuropathic mechanisms of cancer-induced bone pain. The invasion and proliferation of cancer cells result in the damage of primary nociceptive fibers as well as NGF-induced abnormal neuronal sprouting, eventually leading to pain. (B) Inflammatory mechanisms of cancer-induced bone pain. Cancer cells and their surrounding stromal cells, such as immune cells release pro-inflammatory cytokines and chemokines, resulting in the activation of primary nociceptive fibers. (C) Bone-specific mechanisms of cancer-induced bone pain. Tumor-induced PTHrP stimulates the expression of RANK-L on osteoblasts, which activates the RANK receptor and facilitates osteoclastogenesis and bone degradation. Growth factors stored in bone are released after bone degradation, and then promote tumor growth. These processes form the “vicious cycle” in bone remodeling and cause a greater bone degradation and worsening pain. Additionally, the highly acidic environment during bone resorption can also activate nociceptive fibers.

#### **1.2.4 Treatment of chronic cancer pain & limitations**

Current treatment of cancer-induced bone pain primarily follows the guidelines for cancer pain relief developed by the World Health Organization [35]. These guidelines describe a three-step treatment progression from non-opioids (e.g. non-steroidal anti-inflammatory drugs (NSAIDs)), then mild opioids (e.g. codeine), to strong opioids (e.g. morphine) accompanied with additional supplementation (e.g. bisphosphonates and anti-depressants) to treat the worsening pain.

Although this three-step treatment strategy is effective in most of the cases, the severe dose-limiting side effects associated with these analgesic therapies cannot be ignored and have largely compromised the quality of patients' life. NSAIDs, used for treating mild cancer pain, are typically associated with adverse gastrointestinal, cardiovascular, hepatic and renal effects [36]. In some cases, central nervous system side effects, such as headaches, tinnitus and dizziness, may also be induced by this type of medication [37]. Opioids, as the most effective analgesics in the treatment of moderate and severe pain, are commonly associated with respiratory depression, constipation, nausea, vomiting, sedation, and cognitive effects [38]. As opioids possess a strong abuse potential, long-term use of opioids is also associated with an increased risk of dependence and addiction. Additionally, in both human and animal studies, chronic use of opioids was found to increase the potential of bone destruction and tumor growth, which is counterproductive to the management of bone cancer pain. Lastly, opioid tolerance is another side effect that is constantly presented in the patients who use

opioids chronically. Patients have to increase the doses of opioids to counteract this effect, which further increases the potential of having those side effects described above. Taken together, the dose-limiting side effects associated with current treatment of bone cancer pain point out the importance of developing novel analgesics without producing dose-limiting side effects or at least to a lesser extent.

### **1.3 Opioid epidemic**

#### **1.3.1 Prevalence of opioid epidemic**

Chronic pain is among the most common reasons for people to seek medical care due to its disabling effects on patients' daily activities and well-being. Opioids, the currently most effective analgesics, are frequently prescribed to manage this distressing syndrome. In 2014 alone, approximate 250 million prescriptions for opioid pain relievers were dispensed [39,40]. Most of these prescribed opioids (~65%) were applied for short-term therapy (less than 3 weeks), and nearly 10 million Americans were using these prescribed opioids chronically [41,42]. Although opioids can exert a powerful analgesic effect on acute pain, current evidence does not seem to support the effectiveness of chronic opioid therapy in the treatment of chronic pain [43]. More severely, as opioids possess a highly addictive potential, this wide prescription of opioids has led to a severe opioid epidemic in North America, particularly in the United States. According to the 2017 National survey on Drug Use and Health, it was estimated that over 11 million Americans misused prescription opioids and approximate 2 million of them had use disorders [44]. This increased prevalence of opioid abusers has also

caused a striking increase in emergency room visits and overdose death for non-medical use of opioid pain relievers [45,46]. Unfortunately, the opioid epidemic does not seem to be ameliorated within a short period of time [46], resulting in the urgent need of developing non-addictive opioid pain relievers and treatments to prevent opioid addiction.

### **1.3.2 Current treatment of opioid abuse and addiction**

Currently, three classes of medications have been applied to treat opioid abuse and addiction (Table 1) [47]. The first class is the  $\mu$ -opioid receptor (MOR) antagonists, including naloxone and naltrexone. These two drugs are used to treat opioid overdoses and prevent patients from relapse after detoxification respectively. The second class includes methadone and buprenorphine, which are full/partial agonists targeting MORs, respectively. Different from regular prescription opioids or illicit opioids that produce strong rewarding effects by rapid entry into brain, these two agonists distribute in the brain very slowly, thus largely limiting their rewarding effects. At the same time, these two medications also display slow dissociation and clearance from MORs, which, therefore, are useful in the reduction of the craving and withdrawal symptoms during detoxification treatment. The last class of medications used to treat opioid use disorder is  $\alpha$ 2-adrenergic receptor agonist, including lofexidine and clonidine [47,48]. Due to its ability to cause sedation and relaxation, it has been used to treat the withdrawal symptoms in opioid-dependent patients. To date, however, no medication can directly attenuate the rewarding and motivational effects of opioids. As opioid-

induced reward is the initial but critical step towards opioid addiction, developing a medication to prevent this process is essential to deal with the current opioid epidemic.

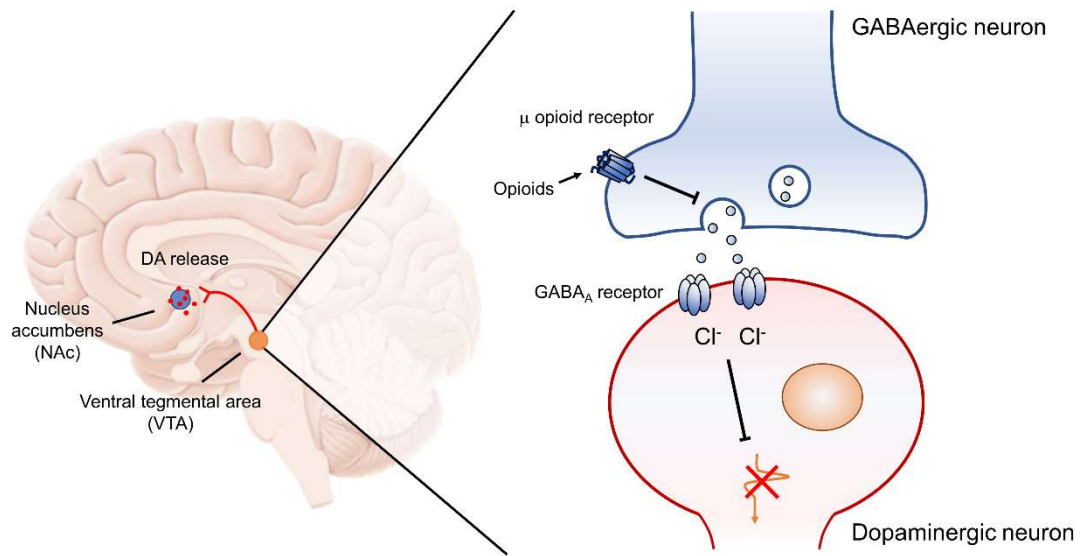
**Table 1 Current medications for opioid use disorders**

Classification	Medication	Medical use(s)
Antagonists at $\mu$ -opioid receptor	Naloxone	Reversing opioid overdoses
	Naltrexone	Preventing from relapse after detoxification
Agonists at $\mu$ -opioid receptor	Methadone/Buprenorphine	Reducing craving and withdrawal symptoms
Agonist at $\alpha$ -adrenergic receptor	Lofexidine	Reducing the symptoms of opioid withdrawal

### 1.3.3 Mechanisms of opioid reward – Two-neuron model

Reward is a CNS process that is induced when natural drives of an individual, such as the desire to eat, drink or mate has been satisfied [2]. From an evolutionary perspective, this feeling of satisfaction guides individuals to perform specific behaviors continually that benefit their survival and reproduction [2]. Current studies identified that ventral tegmental area (VTA) and nucleus accumbens (NAc) are two key brain loci belonging to the mesolimbic circuitry that play a critical role in processing reward-associated stimuli, such as food, water, sex and social dominance [49]. The dopaminergic neurons located in the VTA are

thought to directly encode reward or a reward prediction signal by producing a rapid, phasic dopamine release in NAc (Figure 2) [49]. However, the activity of these dopaminergic neurons is physiologically suppressed by tonic GABA input from VTA GABAergic neurons and other GABAergic neurons from the rostromedial tegmental nucleus (RMTg), NAc and ventral pallidum (Figure 2) [49,50]. Opioids suppress this GABAergic tone by either direct hyperpolarization of the neurons or inhibition of neurotransmitter release, which eventually disinhibits the activity of dopaminergic neurons and allows for dopamine release (Figure 2) [50]. This canonical two-neuron model is commonly thought, at least partially, as the neuronal mechanism controlling opioid-induced reward. Although this model provides a concise way to explain the process of opioid-induced reward, it has been demonstrated to be too simplified and does not include other molecular pathways and systems that also play critical roles in opioid reward, such as endogenous cannabinoid system.



**Figure 2 Two-neuron model of opioid-induced reward**

The GABAergic neuron and dopaminergic neurons form the basic pathway in ventral tegmental area and mediates opioid-induced reward.

## **1.4 Endogenous cannabinoid system**

Over the past 30 years, the endogenous cannabinoid system has been identified as an essential neuromodulatory system. This system is named after the plant *cannabis sativa* (where cannabinoids come from) that led to its discovery. From current studies, we know that endogenous cannabinoid system includes three major components – cannabinoid receptors, endocannabinoids, and the enzymes responsible for the synthesis and degradation of endocannabinoids [51].

### **1.4.1 Cannabinoid receptors**

Cannabinoids exert their pharmacological effects by binding to and activating cannabinoid receptors. However, prior to the identification of cannabinoid receptors, cannabinoids, as highly lipid-soluble molecules, were originally believed to work in a similar way as general anesthetics by affecting ion channels, such as GABAA receptor [52]. To date, two primary cannabinoid receptors were identified, which were termed as type 1 cannabinoid receptor (CB1R) and type 2 cannabinoid receptor (CB2R). Both cannabinoid receptors are G protein-coupled receptors and primarily couple to  $G_{i/o}$  proteins. The activation of these receptors can inhibit adenylyl cyclase and some voltage-gated calcium channels while activate certain mitogen-activated protein kinases and inwardly rectifying potassium channels, which produce a wide range of effects on cellular physiology.

CB1Rs are primarily expressed in the central nervous system, especially in cortex, basal ganglia, hippocampus and cerebellum. Activation of CB1Rs in these

brain regions, therefore, can exert diverse central effects, including memory and learning impairment, motor incoordination, catalepsy, hypothermia, antiemetic action and increased appetite [52]. CB1Rs are also expressed in brain reward circuits, such as nucleus accumbens, ventral tegmental area, globus pallidus, prefrontal cortex and basolateral amygdala [53], which are responsible for the modulatory effects of cannabinoids on drug reward and addiction. In addition to their expression in the central nervous system, CB1Rs are also expressed in some peripheral tissues, such as peripheral nerve and adipocytes, and thus can regulate lipogenesis and nociception. At the subcellular level, CB1Rs are mainly located in the cell membrane of axon terminals while sparing the active zone [52,54] although their expression on glial cells were also reported previously [55–57].

CB2Rs, in contrast to CB1Rs, are primarily located in the periphery particularly in lymphoid tissues, such as spleen, tonsils and thymus, as well as lymphocytes. Therefore, administration of CB2R agonists exerts profound effects on immune functions. Recent studies have demonstrated that CB2Rs are also expressed in the central nervous system. By using quantitative real-time PCR, Walter et al. identified the expression of CB2Rs on microglia [58]. In another study, Zhang et al. found that CB2Rs are expressed on the dopaminergic neurons in VTA at both mRNA and protein levels [59], and the activation of these CB2Rs with a selective CB2R agonist significantly alters the membrane potential and inhibits the firing rate, initiation and duration of action potential in VTA dopaminergic neurons. These data suggest that CB2Rs are functionally expressed in the central nervous system.

In addition to the conventional cannabinoid receptors CB1R and CB2R, a large body of evidence has suggested the possible presence of other cannabinoid receptors [60]. The first report indicating the presence of a non-CB1/CB2 receptor came from the study of the mesenteric vasodilator effect of cannabinoids [61]. In this study, the endocannabinoids anandamide and R-methanandamide induced long-lasting vasodilation in the rat isolated mesenteric arterial bed preparation. This effect was not observed in those synthetic cannabinoids with the ability to activate both CB1Rs and CB2Rs. In another study, the elevated anandamide-induced hypotension was found to be blocked by a selective CB1R agonist SR141716 in CB1R-knockout mice, indicating the involvement of a novel cannabinoid-activating receptor [62,63]. Furthermore, administration of SR141716 inhibited milk suckling in newborn CB1R-knockout mice, which also suggests the presence of a non-CB1/CB2 cannabinoid receptor [64].

Among all the potential cannabinoid receptors, GPR55 has attracted the most attention. This receptor was first isolated in 1999 as an orphan GPCR belonging to the class A  $\delta$ -group with a high expression in human striatum [65,66]. In previous studies, the endocannabinoid anandamide was shown to stimulate several downstream signaling events of GPR55, including [<sup>35</sup>S]GTP $\gamma$ S binding and calcium mobilization, but not others including ERK1/2 phosphorylation,  $\beta$ -arrestin recruitment and GPR55 internalization [67]. These data suggest a putative biased agonism of GPR55 by AEA but they may also simply result from the use of different cell systems or assay end points. Similar experiments have also performed with 2-AG, and only [<sup>35</sup>S]GTP $\gamma$ S binding assay displayed positive results while all other

assays failed [67]. Taken together, current evidence is not enough to suggest GPR55 as a cannabinoid receptor.

The transient receptor potential vanilloid type 1 receptor (TRPV1) is another putative cannabinoid receptor. This receptor belongs to the transient receptor potential family of ion channels, which are responsible for the transduction of a wide range of stimuli, such as xenobiotic substances, temperature, endogenous lipids, mechanical and osmotic stimuli (2010 pertwee-venkataharam2007). TRPV1 can respond to capsaicin, an active component of chili peppers and generate the sensation of hot and pungency (2010 pertwee-caterina 1997). It can also be activated by other noxious stimuli, such as heat ( $>43^{\circ}\text{C}$ ) and protons ( $\text{pH}<6.9$ ). To date, a large body of evidence has demonstrated that endocannabinoids, such as AEA but not 2-AG can activate the TRPV1 in human and other animals as a full agonists although the potency of AEA on TRPV1 may vary depending on the functional assays and the pharmacological properties assessed [68]. Furthermore, as the TRPV1 gating by its ligands can be significantly modulated by multiple regulatory events and signals, such as post-translational modifications, allosteric modulation by temperature, acid and membrane potential, the AEA-induced activation of TRPV1 can be increased or decreased [67]. In addition to AEA, several synthetic cannabinoids (e.g. HU-210) and phytocannabinoids (e.g. cannabidiol and cannabigerol) were also shown to act on TRPV1 [69,70]. Overall, current evidence strongly suggests that TRPV1 can be classified as an ionotropic cannabinoid receptor.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor family [71]. Currently, three isoforms of PPARs were identified - PPAR $\alpha$ , PPAR $\beta$  and PPAR $\gamma$ , and they can form functional units as heterodimers with retinoid X receptors when activated [67]. PPARs were identified to bind with fatty acids and their derivatives, so they are commonly believed to be lipid sensors, monitoring local changes in metabolism. The idea that PPARs are putative cannabinoid receptors came from the fact that a number of CB1R/CB2R agonists were found to activate PPARs in in vitro experiments, including endocannabinoids (e.g. AEA and 2-AG), phytocannabinoids ( $\Delta^9$ -THC) and synthetic cannabinoids [67]. However, the potencies of these cannabinoids as PPAR agonists or antagonists are relatively low – approximately 2 orders of magnitude lower compared to conventional cannabinoid receptors [67]. Currently, the best evidence suggesting endocannabinoids are endogenous ligands of PPARs comes from the in vivo study using a model of inflammatory pain [72]. In this study, the behavioral responses induced by the increased endocannabinoids were blocked by local administration of a PPAR $\alpha$  antagonist. Nevertheless, more evidence is required in order to determine whether PPARs are possible cannabinoid receptors.

#### **1.4.2 Endocannabinoids & their synthesis and metabolism**

Immediately after the discovery of cannabinoid receptors, the search for their specific endogenous ligands starts. In 1992, the first endocannabinoid anandamide (AEA, N-arachidonoyl-ethanolamine) was identified from the pig brain

extracts as it is capable to compete with a radiolabeled ligand of cannabinoid receptor [73]. Later, the second endocannabinoid 2-arachidonoyl glycerol (2-AG) was identified [74,75]. More recently, other endocannabinoids have also been discovered, such as 2-arachidonoyl-glycerol ether (noladin ether), N-arachidonoyl-dopamine (NADA), and virodhamine [76–79]. However, the presence of these lipids in different tissues has been debated and their pharmacological properties have not been investigated thoroughly [80]. Thus, AEA and 2-AG are currently considered the primary endocannabinoids of cannabinoid signaling.

AEA and 2-AG exert agonist activity at both CB1 and CB2 receptors. AEA is a partial agonist at both receptors with a higher affinity to CB1Rs than to CB2Rs, while 2-AG is a full agonist with almost equal affinity to both receptors [67]. Different from regular transmitters, AEA and 2-AG are not stored in intracellular vesicles due to their lipophilic properties [80]. Instead, they are synthesized with an “on demand” manner by cleaving the membrane lipid precursors and releasing immediately. The synthesis of AEA relies on several different pathways while it primarily derives from the cleavage of a phospholipid precursor N-arachidonoyl-phosphatidylethanolamine (NAPE) by a specific phospholipase D (NAPE-PLD) [81–83] whose activity is controlled by depolarization or the activation of glutamatergic receptors [84]. In addition, recent studies also proposed another three NAPE-PLD-independent multistep pathway, which are also able to synthesize AEA from NAPE [85]. The biosynthesis of 2-AG can also be mediated via several molecular pathways, but the hydrolysis of 1,2-diacylglycerol (DAG) by the DAG lipases (DAGL $\alpha$  and DAGL $\beta$ ) is the primary pathway particularly in the

brain. Other biosynthetic pathways for 2-AG includes (1) hydrolysis of phosphatidylinositol (PI) and subsequently lyso PI by PLA1 and lyso PI-specific PLC, respectively, and (2) dephosphorylation of arachidonic acid-containing lysophosphatidic acid [85].

Degradation of AEA and 2-AG is mediated by cellular reuptake and subsequent intracellular hydrolysis. Fatty acid amide hydrolyase (FAAH) is the primary enzyme responsible for the hydrolysis of AEA. This protein is highly expressed in the central nervous system and the FAAH-expressing neurons were identified in proximity to CB1R-expressing terminals, indicating the involvement of this enzyme in the inactivation of AEA signaling [86]. 2-AG degradation can be mediated via several different enzymes but the major enzyme responsible for its clearance particularly in central nervous system is monoacylglycerol lipase (MAGL). Indeed, a previous study showed that MAGL is responsible for approximate 85% of the 2-AG-hydrolyzing activity in mouse brain [87]. Other enzymes including ABHD6 and ABHD12 may also involve in the metabolism of 2-AG but to a much less extent compared to MAGL. Interestingly, in contrast to the postsynaptic distribution of FAAH, MAGL is mostly present in presynaptic terminals where CB1Rs locate [88,89]. This distinct distribution of MAGL suggests its specific involvement in 2-AG inactivation near its action site [80].

### **1.4.3 Endogenous cannabinoid system in the treatment of pain**

The analgesic effects of cannabinoids have been evaluated in various types of chronic pain conditions, particularly neuropathic pain and inflammatory pain.

Neuropathic pain is produced by the injury to the nerves in peripheral and central nervous systems [90]. Previous studies have shown that systemic administration of synthetic mixed CB1R/CB2R agonists such as WIN55212-2 can produce analgesic effects in multiple neuropathic pain conditions [91–93]. Also, the application of selective cannabinoid receptor agonists (either CB1R or CB2R agonists) can also effectively attenuate neuropathic pain in several animal models [94–96]. Inflammatory pain is another common chronic pain condition and it is caused by tissue damage and the following inflammatory processes, while different classes of cannabinoids (e.g. mixed CB1R/CB2R agonists, CB1R agonists and CB2R agonists) are still able to effectively alleviate this type of chronic pain condition in various inflammatory pain animal models [97–99]. Overall, current studies indicate cannabinoids are promising and effective medications for the treatment of chronic pain conditions.

The anti-nociceptive effects of cannabinoids have also been investigated in the cancer-induced bone pain. WIN55212-2 is the first one being evaluated, and studies have shown that both systemic and intrathecal administration of this compound can significantly suppress bone cancer pain [100,101]. Other cannabinoids such as CB2R agonists (e.g. AM1241 and JWH015) and endocannabinoids (AEA and 2-AG), were also shown to effectively alleviate bone cancer pain [31,102–107].

#### **1.4.4 Strategies to overcome the central side effects of cannabinoids**

Although cannabinoids have shown great promise in the treatment of chronic pain conditions, the antinociception of cannabinoids is usually accompanied with a variety of adverse effects, including memory and learning impairment, motor incoordination, catalepsy, hypothermia, sedation, hyperphagia and constipation [52]. Many of these adverse effects are associated with the CB1Rs expressed in central nervous system, which largely limit the translational applications of cannabinoids in humans.

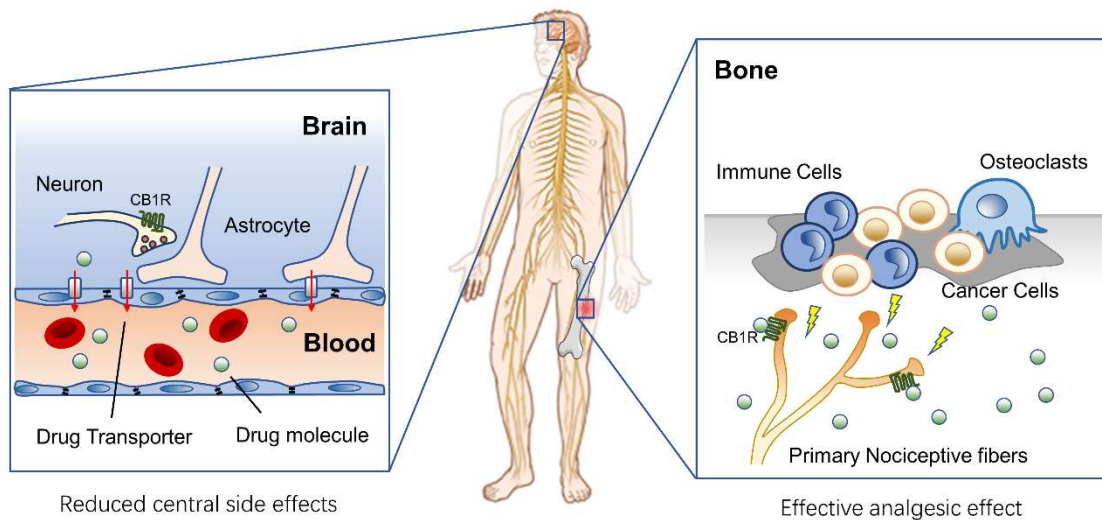
To date, several strategies have been brought to prevent the impairment of cannabinoid-induced central side effects. One strategy is to modulate the cannabinoid system in specific tissues. For example, Khasabova et al. injected (i.paw) AEA, 2-AG or the inhibitors suppressing the degradation of these endocannabinoids in the cancer-bearing legs of the animals, all of which significantly attenuated the mechanical hyperalgesia [104,105]. However, considering the translational applications of this method in patients with bone cancer pain, the performance of injections in specific spots may not be an efficient way to suppress the widespread pain due to the cancer metastasis [15].

Another strategy to reduce the central side effects is to use positive allosteric modulators (PAMs) of CB1Rs rather than typical orthosteric agonists. Allosteric modulators are substances that bind to a receptor and change the response of the receptor by either increasing its affinity to agonists or its ability to be activated by agonists [108]. These unique properties of PAMs enable them to specifically increase the activities of the receptors in the impaired regions, such as

the inflamed tissues where the expression and activities of cannabinoid receptors are increased. Currently, Slivicki et al. and Ignatowska-Jankowska et al. have examined the abilities of two CB1R PAMs in the treatment of neuropathic pain and inflammatory pain, and both studies have shown promising anti-nociceptive effects of PAMs [109,110]. However, as these compounds can penetrate the blood brain barrier, they still possess the potential to produce central side effects particularly when applying together with orthosteric agonists [109,110].

Currently, specific activation of peripheral CB1Rs have been considered as one of the most promising strategies to prevent the occurrence of cannabinoid-mediated central side effects. Importantly, previous studies have discovered that cannabinoid-induced analgesia is primarily mediated through the activation of peripheral CB1Rs, and the expression of CB1Rs in the peripheral tissues is markedly increased under pathological conditions [111–113]. All these studies suggest the application of peripherally restricted CB1R agonists can be an effective way in the treatment of chronic pain. Currently, several peripherally restricted CB1R agonists have been developed and produced robust analgesic effects on different chronic pain conditions with reduced central adverse effects [114–117]. Yet, no studies have investigated the efficacy of peripherally restricted CB1R agonists in the treatment of cancer-induced bone pain. In the present study, we intended to investigate the efficacy of a peripherally restricted CB1R agonist in treating cancer-induced bone pain using a synergistic mouse model (in which the animals have a functional immune system and can better mimic the cancer-

immune interaction in humans) as well as its potential side effects, particularly on central nervous system (Figure 3).



**Figure 3 Strategy to reduce central side effects by using a peripherally restricted CB1R agonist.**

Illustration presents the basic mechanisms about how a peripherally restricted CB1R agonist effectively suppresses bone cancer pain but produces less central side effects. The images of human body and bone were acquired from internet.

#### **1.4.5 Endogenous cannabinoid system in the modulation of opioid reward**

In addition to pain management, the endogenous cannabinoid system has also emerged as promising therapeutic targets in the modulation of opioid reward. CB1R is the primary focus for most of the studies in this field and was found to be altered in some brain regions after chronic opioid exposure, including caudate putamen, hippocampus, nucleus accumbens, midbrain, etc., while the results vary in different reports [118–123]. A large body of evidence has shown that the administration of CB1R antagonists significantly suppresses morphine-induced conditioned place preference (CPP) and heroin self-administration [124–133]. These findings indicate a facilitatory role of CB1R in opioid reward.

AEA is another element in the endogenous cannabinoid system that was intensively investigated. Like CB1Rs, the production of AEA was altered distinctly in different brain regions after chronic opioid treatment [122,134,135]. However, different from CB1Rs, the modulation of AEA production with an FAAH inhibitor URB-597 did not alter the reinforcing effect of heroin or the effects of morphine on VTA dopamine neurons [136,137]. This may suggest the increases in endogenous AEA are not sufficient to modulate opioid reward.

Compared to CB1R and AEA, far too little is known about the modulatory roles of CB2R and 2-AG on opioid reward. Probably due to the extremely low expression of CB2Rs in the central nervous system, the importance of this receptor in the development of mental disorders is frequently undervalued. However, in the past decade, a growing number of studies have gradually revealed the modulatory roles of CB2R on the rewarding processes of multiple addictive substances,

including nicotine, ethanol and cocaine [138–140]. Our lab recently found that acute administration of a selective CB2R agonist JWH015 effectively attenuated morphine-induced dopamine release in nucleus accumbens and CPP, indicating the activation of CB2Rs may inhibit opioid reward [141] while the underlying mechanisms are still largely unclear. Although 2-AG was shown to be regulated in different brain regions after chronic opioid exposure, no study has revealed its role in opioid reward. Recently, several studies have suggested that 2-AG may play a role in the development of drug reward. The first study came from Oleson et al., who found that systemic administration of JZL184, a MAGL inhibitor, significantly facilitated food reward seeking [142]. Later, De Luca et al. showed that systemic injection of 2-AG promoted dopamine release in the NAc Shell [143]. Buczynski et al. discovered that DAGL inhibition suppressed nicotine self-administration [144]. In the present study, we intended to have a comprehensive understanding about how chronic opioid modulates 2-AG and CB2R in reward circuit, particularly in VTA, a key brain region in reward processing, and how these alterations may contribute to chronic opioid-induced reward.

### **1.5 Hypothesis and specific aims**

Based on current literature and our understanding of the endogenous cannabinoid system, we intended to investigate two research questions in this dissertation:

1. Is the use of a peripherally restricted CB1R agonist a promising therapy in the treatment of cancer-induced bone pain without causing central side effects and other adverse effects associated with typical cannabinoids?

Hypothesis: The use of a peripherally restricted CB1R agonist can reduce cancer-induced bone pain without producing central side effects and other adverse effects associated with typical cannabinoids.

Aim 1.1 To examine the analgesic effect of a peripherally restricted CB1R agonist on the spontaneous pain behaviors in a murine model of cancer-induced bone pain.

Aim 1.2 To assess if the peripherally restricted CB1R agonist produces the central side effects that are commonly induced by typical cannabinoids (tetrad effects).

Aim 1.3 To determine the effect of a peripherally restricted CB1R agonist on cancer-induced bone remodeling.

Aim 1.4 To assess if the peripherally restricted CB1R agonist promotes food intake.

Aim 1.5 To assess if the peripherally restricted CB1R agonist induces constipation.

Aim 1.6 To investigate the effect of a peripherally restricted CB1R agonist on tumor growth.

2. How chronic opioid modulates 2-AG and CB2R in reward circuit, particularly in VTA, a key brain region in reward processing, and how these alterations may contribute to chronic opioid-induced reward?

Hypothesis: Chronic opioid exposure alters the production of 2-AG and the expression of CB2Rs in VTA, which contributes to chronic opioid-induced reward.

Aim 2.1 To examine the expression changes of the enzymes that responsible for the synthesis and degradation of 2-AG in VTA after chronic morphine exposure.

Aim 2.2 To assess the changes in 2-AG production in VTA after chronic morphine exposure.

Aim 2.3 To examine the change in expression of CB2Rs in VTA after chronic morphine exposure.

Aim 2.4 To investigate the modulatory effects of 2-AG on chronic morphine-induced reward.

Aim 2.5 To investigate the modulatory effects of CB2Rs on chronic morphine-induced reward.

## **CHAPTER 2: MATERIALS AND METHODS**

### **2.1 In vitro**

#### **2.1.1 Cell culture**

Murine mammary tumor line 66.1 was a kind gift from Dr. Amy M. Fulton [145]. 66.1 cells were cultured in Eagle's minimum essential medium (MEM) with 10% fetal bovine serum, 100 IU-1 penicillin and 100 µg/mL streptomycin and then housed at 37°C and 5% CO<sub>2</sub>. For all assays, cells were counted using a gridded hemocytometer (Hausser Scientific, Horsham, PA).

#### **2.1.2 C-terminal telopeptides of Type I collagen assay**

The serum levels of C-terminal telopeptides of Type I collagen (CTX) were measured by using a CTX assay purchased from Immunodiagnostic Systems (AC-02F1). To collect serum, mice were anesthetized with ketamine/xylazine mix and whole blood was harvested via transcardial puncture. After blood coagulation, serum was isolated by centrifugation and stored at -80°C until testing. CTX assay was conducted according to the manufacturer's instructions.

#### **2.1.3 XTT assay**

XTT cell viability assay (ATCC, Manassas, VA) was performed according to the manufacturer's instructions. Briefly, 66.1 breast cancer cells were plated into a 96-well plate at a density of  $1 \times 10^4$  per well and allowed to grow for 24 hours. Cells were then treated with different concentrations of PrNMI (1 nM – 1 µM) or

vehicle for another 24 hours. After treatment, cells were incubated with activated-XTT solution for 2 hours and read at 475 nm and 660 nm using a plate reader.

#### **2.1.4 Western blotting**

66.1 cells and VTA samples were lysed in the Pierce RIPA buffer (89900, Thermo Scientific, Rockford, IL) with protease inhibitor cocktail (B14002, Bimake, Houston, TX) via ultrasonication, and the protein concentration in the tissue lysates were determined using Pierce BCA protein assay kit (23225, Thermo Scientific, Rockford, IL). Protein samples were resolved on 10% SDS-polyacrylamide gels (Criterion TGX; Bio-Rad, Hercules, CA) and subsequently transferred to a polyvinylidene difluoride membrane (PVDF, Bio-Rad, Hercules, CA). PVDF Membrane was blocked with 5% BSA in Tris-buffered saline containing 0.5% (v/v) Tween-20 (TBST) for 1 hour at room temperature, and then incubated with different primary antibodies, including anti-mouse CB1R (ab23703, Abcam; 1:3,000 dilution, rabbit polyclonal), anti-rat CB1R antibody (1:2000 dilution, rabbit polyclonal), anti-rat DAGL $\alpha$  antibody (1:4000 dilution, rabbit polyclonal), anti-rat MAGL antibody (1:2000 dilution, rabbit polyclonal), anti-GFP antibody (MAB3580, Sigma-Aldrich; 1:5000 dilution, mouse monoclonal), anti- $\alpha$ -tubulin antibody (cp06, Calbiochem; 1:50000, mouse monoclonal), and anti- $\beta$ -actin antibody (ab8226, Abcam; 1:10,000, mouse monoclonal) diluted in TBST containing 3% BSA overnight at 4°C. Anti-rat CB1, DAGL $\alpha$ , and MAGL antibodies are kind gifts from Dr. Ken Mackie. After washing with TBST, the membrane was incubated with appropriate secondary antibodies, including HRP-linked anti-rabbit IgG (7074, Cell

Signaling; 1:10,000 dilution) or HRP-linked anti-mouse IgG (7076, Cell Signaling; 1:30,000 dilution) for 1 hour at room temperature. Membrane was again washed and developed using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA). Bands were detected using GeneMate Blue Lite Autorad films (BioExpress, Kaysville, UT) and quantitated using ImageJ software (Wayne Rasband, NIH, USA). All data were normalized to  $\beta$ -actin or  $\alpha$ -tubulin in each lane.

### **2.1.5 Quantitative real-time polymerase chain reaction**

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed as described previously [146]. RNA was extracted from VTA tissues by using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufactures protocol. Briefly, samples were homogenized in 500  $\mu$ L TRIzol reagent, and then 100  $\mu$ L chloroform was added to each homogenate. After centrifugation, the upper aqueous phase containing RNA was transferred to a new tube and total RNA was subsequently precipitated after adding 250  $\mu$ L isopropanol to the aqueous phase. Following by another centrifugation, the RNA precipitates were washed with 75% ethanol and resuspended in 20  $\mu$ L DEPC-treated water (ThermoFisher, Grand Island, NY). cDNA was generated immediately after RNA extraction by using the Maxima Reverse transcriptase kit (ThermoFisher, Grand Island, NY) according to its manufactures protocol. qRT-PCR analysis was performed by using 5x HOT FIREPol EvaGreen qPCR Mix Plus (08-25-00001, Solis Biodyne, Estonia) on a CFX connect real-time PCR detection system (Bio-rad, Hercules, CA) according to the manufactures protocol. The relative mRNA expression for CB1R and CB2R

genes was normalized to  $\beta$ -actin mRNA level and calculated with the  $\Delta\Delta$ Ct method [147]. The sequences of all specific primers were listed in the table below (Table 2):

**Table 2 The qRT-PCR primers for different gene expression in rats**

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
CB1R	ACCTACCTGATGTTCTGGATTGGG	CGTGTGGATGATGATGCTCTTCTG
CB2R	CTCGTACCTGTTTCATCGGCA	GTATCGGTCAACAGCGGTCA
$\beta$ -actin	TAAGGCCAACCGTGAAAAGATGA	GTACGACCAGAGGCATACAGG

### 2.1.6 Measurement of monoacylglycerol lipase activity

The activity of MAGL in the VTA was measured by using a MAGL activity assay kit (K561, BioVision, Milpitas, CA). Briefly, the VTA tissues were rapidly homogenized in the ice cold MAGL assay buffer. After centrifugation, the supernatant of each sample was collected and assayed using the manufactures protocol. The fluorescence of the samples was measured in kinetic mode (Ex/Em = 360/460 nm) with a multi-mode microplate reader (Synergy 2, BioTek, Winooski, VT). MAGL activity was calculated from a standard curve and the presented data is normalized to total sample protein.

### **2.1.7 Single guide RNA design for CB2 receptor (cnr2) gene editing**

Our strategy to edit the *cnr2* gene was to use a genomic editing technique termed CRISPR/Cas9 [148]. The single guide RNA (sgRNA) that specifically targets *cnr2* gene was designed to match the 5' end of the protein coding sequence that can completely deactivate the function of the whole *cnr2* gene. We identified and extracted the sequence of the *cnr2* gene from the genomic sequence of chromosome 5 from SD rats (AC\_000073.1). To design the sgRNA sequence, we screened for potential off-targets using the sgRNA design program (<http://crispr.mit.edu>). The final sgRNA we acquired to target *cnr2* gene is 5'-GCAAGCCGCCATTGGAGCCGT-3', with a quality score of 92.

### **2.1.8 Plasmid construction**

#### **2.1.8.1 Cloning of sgRNA**

The *cnr2* sgRNA was cloned into the pSpCas9(BB)-2A-GFP plasmid (PX458, #48138, Addgene, Cambridge, MA) that allows for simultaneous expression of the Cas9 nuclease and sgRNA. The plasmid was digested by using the BbsI restriction enzyme (FD1014, Thermo Fisher Scientific, Waltham, MA) and purified after running through a 1% agarose gel. The oligonucleotides for cloning sgRNA was designed according to XXX (*cnr2* forward: 5'-CACCGCAAGCCGCCATTGGAGCCGT-3'; *cnr2* reverse: 5'-AAACACGGCTCCAATGGCGGCTTGC-3') and purchased from Eurofins, Louisville, KY. A 10- $\mu$ M solution of forward and reverse oligonucleotides was annealed in a thermocycler using the following protocol: 37°C for 30 minutes, 95°C

for 5 minutes, with a cooling to 25°C at a rate of 5°C/min. Then, 100 ng of the digested pSpCas9(BB)-2A-GFP plasmid was set up for ligation with 50 nM of the annealed oligonucleotides at 22°C for 5 minutes using Rapid DNA ligation Kit (K1422, Thermo Fisher Scientific). The ligation products were transformed into *E. coli* DH5α competent bacteria (C2987; New England Biolabs, Ipswich, MA) according to manufacturer's instructions. A 100% efficiency was observed for the insertion of gRNA sequences into the pSpCas9 (BB)-2A-GFP plasmid. Plasmids were purified from DH5α *E. coli* using the NucleoBond Xtra Maxi kit (740414; Macherey-Nagel, Düren, Germany) and verified by Sanger sequencing (Eurofins). Similar steps were applied to the cloning of sgRNA to the pL-CRISPR-EFS-tRFP plasmid (#57819, Addgene, Cambridge, MA) but at the Esp3I sites (FD0454, Thermo Fisher Scientific, Waltham, MA). Packaging lentivirus with pL-CRISPR-EFS-tRFP plasmid was conducted by VectorBuilder Inc. (Chicago, IL).

#### **2.1.8.2 Construction of target plasmid expressing rat CB2R gene**

The coding sequence corresponding to rat CB2R gene was cloned between the XbaI and BamHI restriction sites in the pLenti-CMV-GFP-puro backbone (#17448, Addgene, Cambridge, MA). SD rat genomic DNA was purified using the Quick-DNA mini prep kit (11-397, Genesee). The coding sequence for CB2R gene was amplified through PCR using HD advantage polymerase (639241, Clontech) and the primer pair: 5'-AGACACCGACTCTAGATGGCGGGATGCCGGGAGC-3' and 5'-GGCGACCGGTGGATCGCAATTGGAGCAGCCTGGTG-3'. PCR products were subsequently recombined in pLenti-CMV-GFP-puro plasmid using the In-

Fusion PCR Cloning Kit (638909, Clontech). Successful recombination was verified by Sanger sequencing (Eurofins).

### **2.1.9 Culturing and transfection of catecholamine A differentiated (CAD) cells**

Mouse neuron derived CAD (#08100805, ECACC) were grown in standard cell culture conditions, 37°C in 5% CO<sub>2</sub>, as previously described [149]. All media was supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin sulfate from 10,000 µg/ml stock. CAD cells were maintained in DMEM/F12 media. Cells were transfected using 1mg/ml Polyethyleneimine (PEI), linear, M.W. 25,000 (#AA43896-03, VWR) complexed with pLenti-CMV-GFP-puro-CB2R plasmid (ratio 1:3, w/v, plasmid/PEI) prepared in OptiMEM (#31985070, Thermo Fisher Scientific). Under these conditions, transfection efficiencies were >95%. To test the on-target efficacy of the pL-CRISPR-EFS-GFP-cnr2 gRNA targeting strategy, the CRISPR plasmid (with or without gRNA) was co-transfected with the CB2R-expressing plasmid. All experiments were performed between 48 h and 72 h after transfection.

### **2.1.10 Tracking of indels by decomposition analysis**

To evaluate the CRISPR/Cas9 editing efficiency *in vivo*, an analysis method termed Tracking of Indels by Decomposition (TIDE) was used [150]. Genomic DNA was extracted from VTA tissues using Purelink Genomic DNA mini kit (K182001, Thermo Fisher Scientific) following manufacture's instruction. PCR amplicons

spanning the CRISPR/Cas9 editing sites were generated using CloneAmp HiFi PCR premix (639298, TAKARA, Mountain View, CA) with the following primer pair: CB2R-TIDE-forward (5'-CTGGGTTTCAAAGGGAAGTTTCAG-3') and CB2R-TIDE-reverse (5'-CCATGAGCGGTAGGTAGGAG-3'). PCR products were purified by running through a 1% agarose gel and then sequenced by Eton Bioscience Inc. (San Diego, CA). The sequence trace data of each PCR product was analyzed using the online TIDE program (<http://tide.nki.nl>). All parameters in the TIDE program were set as default.

## **2.2 In vivo**

### **2.2.1 Animals**

All procedures were approved by the University of Arizona Animal Care and Use Committee and conform to the Guidelines by the National Institutes of Health and the International Association for the Study of Pain. Adult female BALB/cAnNHsd mice (18-20 g; Harlan, Indianapolis, IN) and male Sprague Dawley rats (7-8 weeks old; Envigo, Indianapolis, IN) were maintained in a climate-controlled room on a 12-hour light-dark cycle and allowed food and water ad libitum. Mice were housed five per cage for all experiments. Rats were housed three per cage for all experiments except for those received guide cannula implantation, which were housed individually. Rats were monitored on days 1-3 after cannula implantation. Mice were monitored on days 0, 7, 10 and 14 of the study for clinical signs of rapid weight loss and signs of distress after cancer inoculation.

### 2.2.2 Drug treatment

PrNMI, a peripherally restricted CB1R agonist, was acquired from our collaborator Dr. Igor Spigelman. SR141716, a CB1R antagonist, was purchased from RTI International (Research Triangle Park, NC). SR144528, a CB2R antagonist, and JWH015, a selective CB2R agonist were purchased from Tocris (Bristol, UK). MJN110, a selective MAGL inhibitor, was purchased from Cayman Chemical (Ann Arbor, MI). All these compounds are dissolved in a vehicle solution composing of 10% dimethyl sulfoxide, 10% Tween-80 and 80% saline for injections in animals (10 mL/kg, i.p. and s.c. for mice; 1 mL/kg, i.p. for rats). Morphine sulfate was obtained from the NIDA Drug Supply program and was dissolved in saline for injection. Acute studies applied one injection of PrNMI (0.1, 0.3, 0.6 or 1 mg/kg, i.p.) or vehicle. Chronic studies consisted of once-daily injection of PrNMI (0.6 mg/kg, i.p., every day [q.d.], days 7-14) or vehicle after femoral inoculation. Separate sets of mice were treated with selective CB1R antagonist SR141716 (RTI International, Research Triangle Park, NC) or CB2R antagonist SR144528 (Tocris, Bristol, UK) (1 mg/kg, i.p., q.d., 10 minutes prior to PrNMI or 5 µg per 5 µL (lumbar puncture, l.p.), day 14, 30 minutes prior to the behavioral testing). Chronic morphine administration in rats was performed by intraperitoneal injections of morphine sulfate to rats at a dose of 5 mg/kg twice daily for 5 consecutive days [151]. For conditioned place preference testing, rats were received a total of 5 injections of morphine at a dose of 10 mg/kg intraperitoneally.

### **2.2.3 Intramedullary implantation of 66.1 breast cancer cells**

To establish CIBP in mice using a syngenic model [152], an arthrotomy was performed as previously described [153]. Briefly, mice were anesthetized with 80 mg/kg ketamine + 12 mg/kg xylazine (in 10 mL/kg, intraperitoneally (i.p.) injection volume) and the condyles of the right distal femur were exposed. A hole was drilled at the intercondylar notch and into the medullary canal to create an initial core pathway. A placement needle was then inserted to make the final pathway into the bone. Proper placement of the needle was confirmed by radiograph (UltraFocus, Faxitron Bioptics, Tucson, AZ). Next,  $8 \times 10^4$  66.1 cells in 5  $\mu$ L complete MEM or 5  $\mu$ L complete MEM alone (as a control) was injected into the intramedullary space of the femur and the injection sites were sealed with bone cement. Muscle and skin were closed in separate layers with 5-0 Vicryl suture and wound autoclips, respectively. All mice were monitored for anesthesia during the surgery to ensure that no whisker movement or toe pinch response was presented. Gentamicin (8mg/kg, 10 mL/kg volume, subcutaneously (s.c.)) was given to all mice after surgery in order to prevent infection. Staples were removed 7 days after surgery.

### **2.2.4 Behavioral testing protocols**

#### **2.2.4.1 Acute and chronic behavioral testing of spontaneous pain**

Spontaneous pain-related behaviors, flinching and guarding, were recorded as previously described [153]. Flinching was characterized by the lifting and rapid flexing of the ipsilateral hindpaw not associated with walking or other movement. Guarding was characterized by fully retracting the ipsilateral hindpaw under the

torso. These two behaviors were observed for 2 minutes during a resting state after a 30-minute acclimation period. The number of flinches and the time the hindpaw was retracted during the 2-minute period were recorded. Both flinching and guarding of the cancer-bearing limb are best described as measurements of ongoing pain that is reflective in patients with bone cancer who protect affected limbs [154,155]. Guarding and spontaneous flinching are behaviors observed in which there is, no to very little, contact with a ground surface and becomes more progressive with time (days after femur inoculation). Acute behavioral testing: Baseline behaviors of spontaneous pain were recorded seven days after surgery. Mice were then separated into treatment groups and received a single dose of drug. After drug administration, mice were tested over a 24-hour time course until their pain behaviors returned to baseline. Chronic behavioral testing: Spontaneous pain behaviors were assessed before surgery (baseline). Mice then received treatment at the same time of each day during day 7-14. Spontaneous pain behaviors were measured 3 hours after treatment on days 7, 10 and 14, based on the time of peak effect determined by the acute studies.

#### **2.2.4.2 Open field testing**

An open field test was used to investigate the potential sedative effect of PrNMI. The open-field arena (33 cm x 28 cm x 33 cm) is a white box with an open top and a black floor. A rectangle (16.5 cm x 14 cm) was marked in the center of the field. Sessions began by placing the mouse in the center rectangle and ended after 5 minutes. A consistent white noise (~55 dB) and a dim lighting (~24 lux) were

applied during the test. The entire session was recorded by a video camera mounted 1.5 m above the floor. The tracking of mouse movement was realized by analyzing the testing videos with an open-source tracking software, EthoWatcher [156].

#### **2.2.4.3 Rotarod testing**

A rotarod test was used to determine the motor effect of PrNMI. Four days before testing, naïve mice were trained to acclimate to the rotating rod (LE8505 Rota-Rod, Panlab Harvard Apparatus, Spain) at a speed of 10 rpm. A maximal cut-off time of 180 seconds was used to prevent exhaustion. On the day of testing, mice were baselined and reevaluated 3 hours after treatment.

#### **2.2.4.4 Rectal temperature testing**

Animal rectal temperature was measured by using a thermistor probe (Thermoworks, American Fork, UT) to investigate the hypothermia induced by PrNMI. On the testing day, the rectal temperature was measured before treatment and 3 hours after treatment.

#### **2.2.4.5 Ring immobility testing**

As CB1R agonists cause catalepsy in animals [157], a ring immobility test was used to determine the cataleptic effect of PrNMI, as described previously [158]. Briefly, mice were placed on a horizontal metal ring (5.5 cm diameter) attached to a ring stand at a height of 16 cm. Each mouse was observed for 5 minutes and the

sum of time it remained motionless was counted. The criterion for immobility was the absence of all voluntary movements including snout and whisker movements, but the movements associated with breathing were excluded. Immobility is described as the percentage of the 5-minute period in which the mouse was motionless.

#### **2.2.4.6 Conditioned place preference**

In order to mimic the chronic morphine model, the procedure of conditioned place preference (CPP) was modified from our previous studies [141,159]. Rats were preconditioned to a three-chambered CPP apparatus (San Diego Instruments, San Diego, CA) for 15 minutes to acquire their baseline preference for the two side chambers. Only rats that showed no obvious preference (<80% of the total time) or aversion (>20% of the total time) to any side chambers were used and randomly assigned for further conditioning experiments over the next 5 days. In the morning session of the first conditioning day, rats were pretreated with MJN110 (5 mg/kg, i.p.), JWH015 (3 mg/kg, i.p.) or vehicle. Thirty minutes after the pretreatment, animals were then injected with morphine (10 mg/kg, i.p.) or saline and confined to one side chamber (drug-paired chamber) for 15 minutes. In the afternoon session, all rats were pretreated with vehicle followed by an injection of saline and paired with another side chamber (non-drug paired chamber) as a control. The same procedures were repeated on the conditioning days 2-5 while the morning and afternoon sessions in days 2 and 4 were inverted in order to counter-balance the time effect. On the test day (day 6), rats were allowed to

explore all chambers of the CPP box freely for 15 minutes and the total time they spent in each side chamber was recorded to determine their chamber preference. The chamber preference is presented as CPP score, which is calculated as below:  
CPP score = Time spent in drug-paired chamber on the test day – Time spent in drug-paired chamber on baseline day

### **2.2.5 Radiography**

A digital Faxitron machine (UltraFocus, Faxitron Bioptics, Tucson, AZ) was used to acquire radiographs of mice anesthetized with ketamine/xylazine before surgery and day 14 after surgery. Bone loss was rated by 3 blinded observers trained in scoring animal radiographs according to the following scale: 0 = normal bone, 1 = 1 to 3 radiographic lesions indicating bone loss, 2 = 4 to 6 radiographic lesions indicating bone loss, 3 = full-thickness unicortical bone loss indicating unicortical bone fracture and 4 = full-thickness bicortical bone loss indicating bicortical bone fracture. Observer scores for each bone on day 14 were averaged.

### **2.2.6 Measurement of body weight and body composition**

The body weight of mice in bone cancer pain studies was measured on day 0 before surgery, days 7 and 14 post-surgery using an animal weight scale. The animal body composition including fat and lean contents, was assessed using dual-energy x-ray absorptiometry (DEXA) by a digital Faxitron machine (UltraFocus, Faxitron Bioptics, Tucson, AZ) on day 14 post-surgery.

### **2.2.7 Assessment of defecation**

The ability of mice to defecate was used to evaluate if PrNMI can induce constipation. After PrNMI treatment, the mice were placed in an open-field arena (33 cm x 28 cm x 33 cm) for 5 minutes. The number of fecal pellets produced within this period of time was recorded.

### **2.2.8 in vivo microdialysis for endocannabinoids**

Rats were first anesthetized with ketamine (80 mg/kg)/ xylazine (10 mg/kg) mix and secured in a stereotaxic apparatus. A unilateral microdialysis guide cannula (20 mm, MAB 2/6/9.20.G, SciPro, Sanborn, NY) was implanted into the ventral tegmental area (VTA) according to the Paxinos and Watson atlas (2007) [160]: AP -5.9 mm, ML +0.5 mm, and DV -8.2 mm from bregma. The guide cannula was fixed in place with skull screws and dental cement. All rats were injected with the antibiotic gentamicin (8 mg/kg, s.c.) to prevent from infection.

Microdialysis experiments were conducted one day after chronic morphine treatment. The performance of microdialysis was modified from previous studies [141,161,162]. At 2 hours prior to sample collection, rats were anesthetized with 2% isoflurane and a microdialysis probe with 1 mm PES membrane and 15kD cut-off (MAB 6.20.1) was placed and secured in the guide cannula. Artificial cerebral spinal fluid (aCSF) containing 10% (w/v) hydroxypropyl- $\beta$ -cyclodextrin (Cayman Chemical, Ann Arbor, MI) was then used to perfuse the microdialysis probe and equilibrate within the brain tissue at a flow rate of 1  $\mu$ L/min. Following a 2-hour baseline period, all rats were injected with morphine (5 mg/kg, i.p.) and dialysate

samples were collected every 30 minutes for a total of 4 hours. Collected samples were frozen in dry ice after each 30-min interval and subsequently stored at -80°C until further analysis. After experiments, rats were sacrificed, and their brains were harvested and fixed in 10% formalin solution. Coronal slices of the VTA with a thickness of 40  $\mu\text{m}$  were sectioned on a cryostat. The placement of the guide cannulas was verified visually and only those rats with correct cannula placement were used for final analysis.

### **2.2.9 Quantification of endocannabinoid contents in microdialysates**

Analysis of 2-AG and AEA contents in microdialysates was performed by the University of Arizona Cancer Center Analytical Chemistry Core on an Ultivo triple quadrupole mass spectrometer combined with a 1290 Infinity II UPLC system (Agilent, Palo Alto, CA). Samples for analysis were prepared by mixing 10  $\mu\text{L}$  internal standard solution (a mixture of d4-AEA and d4-2-AG in acetonitrile) to 20  $\mu\text{L}$  microdialysate and then centrifuged at 15,800  $\times g$  for 5 minutes at 4°C. The supernatant was transferred to autosampler vials and 5  $\mu\text{L}$  was injected for analysis. Chromatographic separation was achieved using an isocratic system of 21% 1mM ammonium fluoride and 79% methanol on an Acquity UPLC BEH C-18 1.7  $\mu\text{m}$  2.1x100 mm column (Waters, Milford, MA) maintained at 60°C with a flow rate of 400  $\mu\text{L}/\text{min}$ . After each injection the column was washed with 90% methanol for one minute and then re-equilibrated for 5 minutes prior to the next injection. The mass spectrometer was operated in electrospray positive mode with a gas temperature of 150°C at a flow of 5 L/min, nebulizer at 15 psi, capillary voltage of

4500V, sheath gas at 400°C with a flow of 12L/min and nozzle voltage of 300V. The transitions monitored were: m/z348.3 → m/z287.3 (d0-AEA), m/z352.3 → m/z287.4 (d4-AEA), m/z379.3 → m/z287.2 and 269.2 (d0-2-AG), and m/z348.3 → m/z287.2 and 296.1 (d4-2-AG). As 2-AG is reported to be relatively unstable and can rapidly convert to 1-AG, the 2-AG and 1-AG peak areas were combined for all analyses in the present study [135]. The quantification of AEA and 2-AG was achieved by using calibration curves, which were prepared by serial dilution of AEA and 2-AG stock solutions in 80% acetonitrile. The stock solutions of AEA, 2-AG, d4-AEA and d4-2-AG were purchased from Cayman Chemical (Ann Arbor, MI).

#### **2.2.10 Stereotaxic injection of CRISPR/Cas9 plasmids in VTA**

For in vivo transfection, the plasmids pSpCas9(BB)-2A-GFP-cnr2-gRNA or pSpCas9(BB)-2A-GFP-control-gRNA were diluted to 0.5 µg/µL in 5% sterile glucose solution. Then, Turbofect in vivo transfection reagent (R0541; Thermo Fisher Scientific) was added following manufacturer's instructions.

Rats were anesthetized with ketamine (80 mg/kg)/ xylazine (10 mg/kg) mix and secured in a stereotaxic apparatus. Then, 0.5 µl of the plasmid solution containing either cnr2-CRISPR/Cas9 plasmid or control plasmid was directly microinjected into each side of the VTA over 5 min (Coordinate: AP -5.9 mm, ML ±0.5 mm, and DV -7.8 mm from bregma). After injection, all rats were given prophylactic antibiotic gentamycin (8 mg/kg, s.c.) and allowed to recover for two weeks.

## **2.3 Ex vivo**

### **2.3.1 VTA tissue collection**

At 1 hour after the last morphine or saline injection, rats were anesthetized with ketamine (80 mg/kg)/ xylazine (10 mg/kg) mix and transcardially perfused with 1x phosphate buffered saline (pH 7.4). The brains from these animals were carefully removed and the VTA tissues were rapidly dissected on ice according to the Paxinos and Watson atlas (2007) [160]. Immediately after tissue harvest, the VTA samples were snap frozen in liquid nitrogen and subsequently stored at -80°C until they were used for western blotting, qRT-PCR and MAGL activity assay.

## **2.4 Statistical Analysis**

Two-way ANOVA with Tukey's multiple comparisons test was used to analyze acute and chronic behavioral studies of spontaneous pain, the studies of food intake (body weight) and endocannabinoid production (time effect); Two-way ANOVA with Sidak's multiple comparisons test was used to analyze the studies of endocannabinoid production (group difference); Kruskal-Wallis test with Dunn's multiple comparisons test was used to compare the radiograph results; One-way ANOVA with Dunn's multiple comparisons test was used to analyze the studies of central side effects (rotarod, open field testing, rectal temperature testing and ring immobility testing), bone remodeling (CTX assay), food intake (fat and lean mass), constipation (defecation), tumor growth (XTT assay), opioid reward (CPP for MJN110 and JWH015); Unpaired t test was used to compare the protein

expression in western blotting (DAGL $\alpha$ , MAGL, CB1R and GFP), qRT-PCR (CB1R and CB2R), MAGL activity assay, and CPP for CB2R-CRISPR. All data are presented as mean  $\pm$  standard error of the mean (SEM) or median  $\pm$  interquartile range and a value of  $P < 0.05$  was accepted as statistically significant. GraphPad Prism 7.0 and 8.0 (Graph Pad Inc, San Diego, CA) were used to perform statistical analyses and generate plots. Power analyses were performed on cumulated data using G\*Power 3.1 software to estimate the optimal numbers required.

## **CHAPTER 3: TARGETING PERIPHERAL CB1 RECEPTORS WITH A PERIPHERALLY RESTRICTED CB1R AGONIST IN THE TREATMENT OF CANCER-INDUCED BONE PAIN**

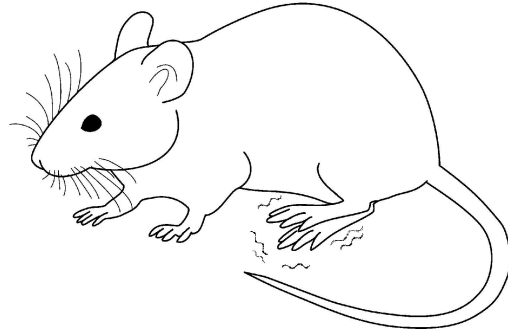
### **3.1 Acute administration of PrNMI attenuates cancer-induced bone pain**

To examine our hypothesis that the use of a peripherally restricted CB1R agonist can effectively reduce cancer-induced bone pain, we investigated the analgesic effect of a peripherally restricted CB1R agonist, PrNMI, in a murine model of cancer-induced bone pain [116]. To establish the model, 66.1 murine breast cancer cells were inoculated into the right femur of BALB/c mice following by sealing the injection sites with bone cement. The cancer-induced bone pain in the mice was assessed by observing the spontaneous pain behaviors, flinching and guarding (Figure 4 A and 5 A). Before surgery, no mouse presented with any spontaneous pain behaviors (Figure 4 B and 5 B). However, seven days after femoral inoculation, mice injected with 66.1 cancer cells displayed a significant amount of flinching and guarding, which are behavioral signs of spontaneous pain (Figure 4 B and 5 B). A single injection of PrNMI (0.1, 0.3, and 0.6 mg/kg, i.p.) resulted in a significant, time-related reduction of flinching but not guarding in a dose-dependent manner (Figure 4 B and 5 B). This suppression of flinching started 1-hour post-injection and persisted for at least 5 hours (Figure 4 A). The maximum

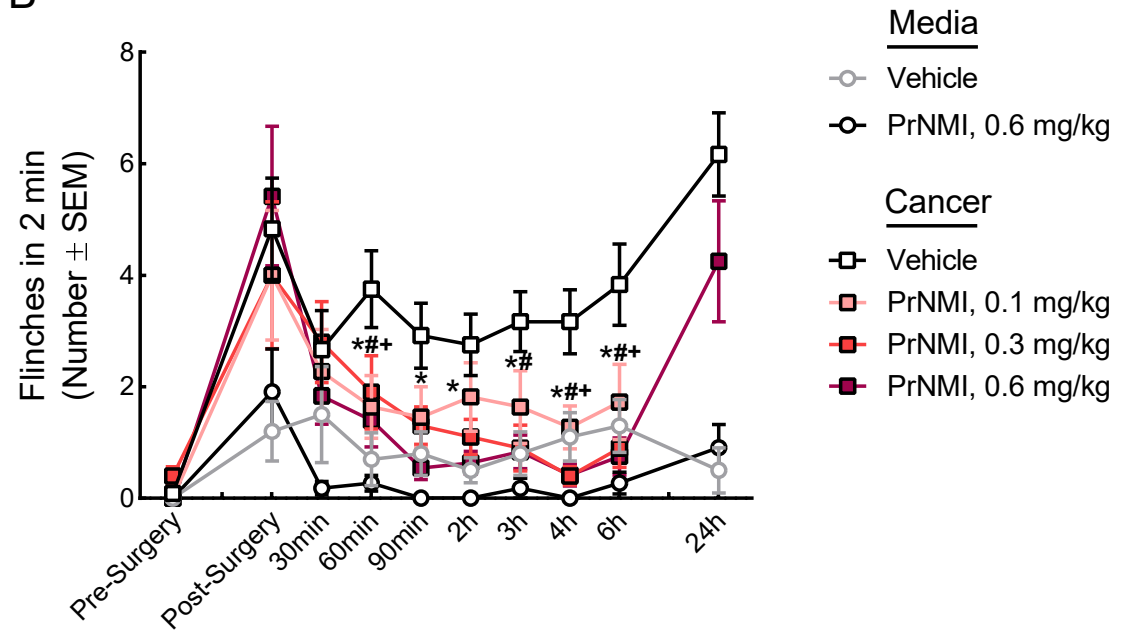
efficacy of PrNMI in the inhibition of spontaneous flinching is ~75% with an ED<sub>50</sub> of 0.077 mg/kg (Figure 4 C).

A

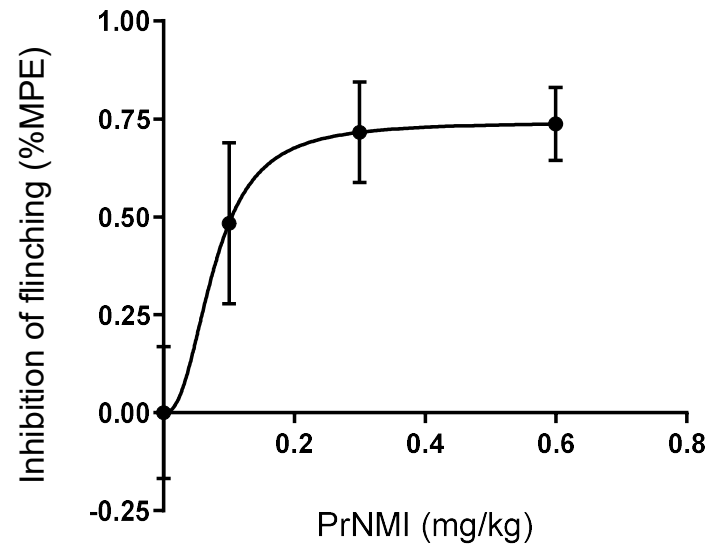
# Flinching



B



C Dose Response Curve

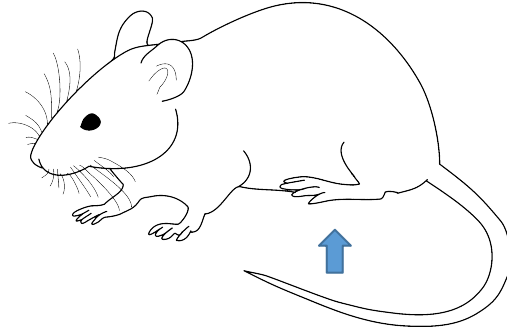


**Figure 4 Acute administration of PrNMI attenuates pain-induced spontaneous flinching in a cancer-induced pain model**

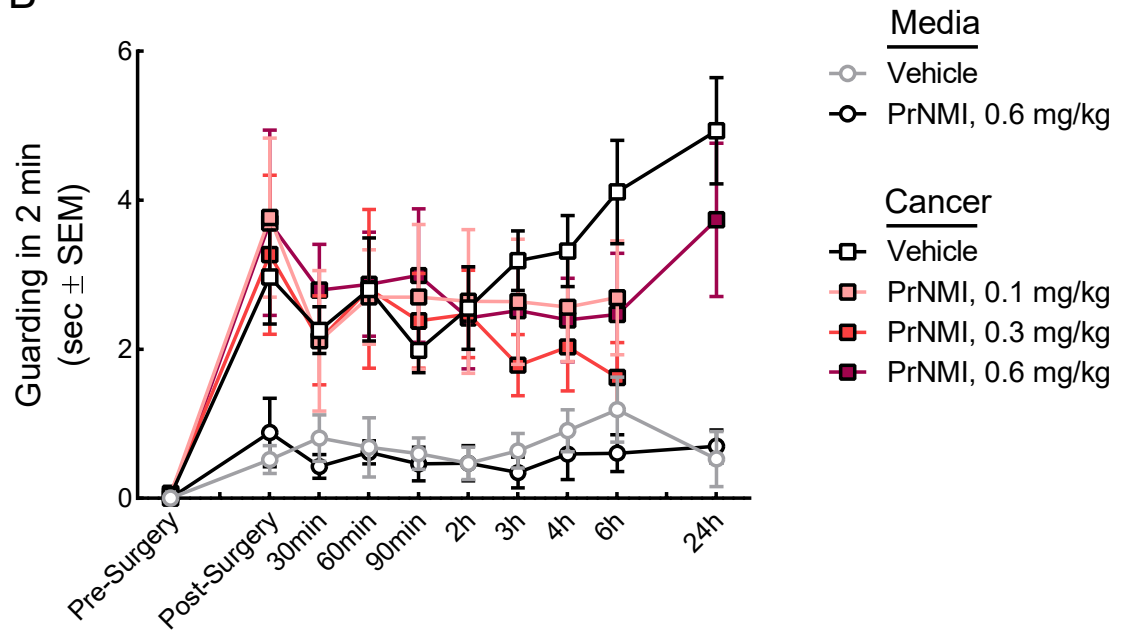
On day 7 after femoral inoculation with 66.1 breast cancer cells or cell-free media, animals were treated with PrNMI (0.1, 0.3, or 0.6 mg/kg, i.p.) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg, i.p.), and spontaneous pain behavior flinching was recorded in a 2-minute period at various time points. (A) A schematic diagram for spontaneous flinching in mice. (B) Spontaneous flinching was significantly reduced by PrNMI compared with animals that received vehicle. No significant difference was observed in media-only control animals between PrNMI-treated and vehicle-treated groups. (C) A dose response curve was made by using the data at 3 hours post-PrNMI injection when PrNMI reached its peak effect on suppressing spontaneous flinching. MPE, maximum possible effect. \* $p < 0.05$ , 0.6 mg/kg PrNMI vs. vehicle; # $p < 0.05$ , 0.3 mg/kg PrNMI vs. vehicle; + $p < 0.05$ , 0.1 mg/kg PrNMI vs. vehicle. Values represent the mean  $\pm$  SEM,  $n = 10-12$  per group.

A

# Guarding



B

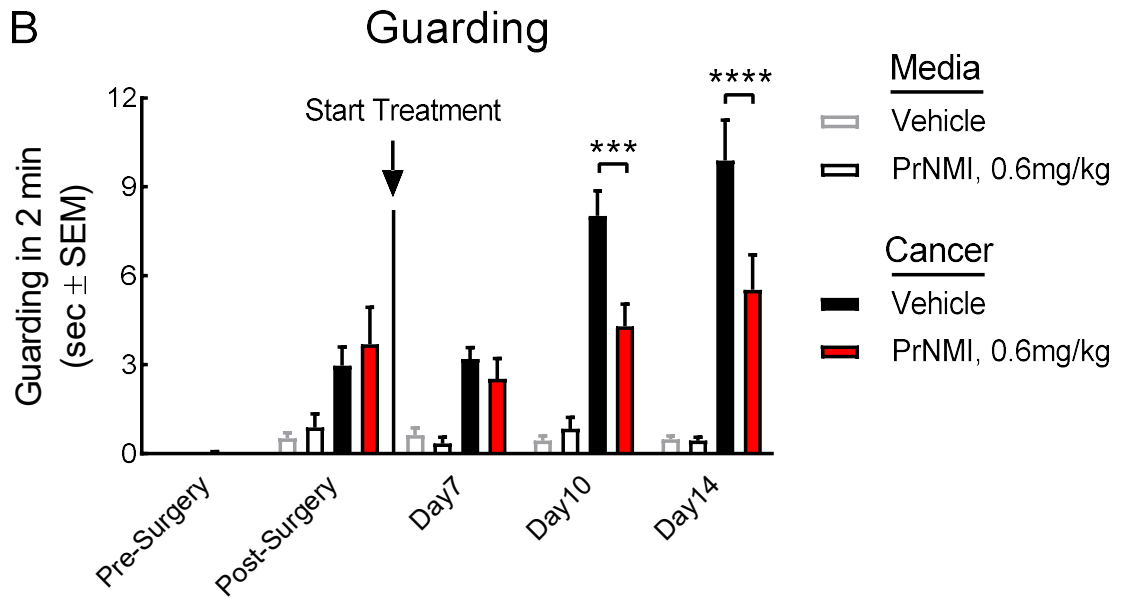
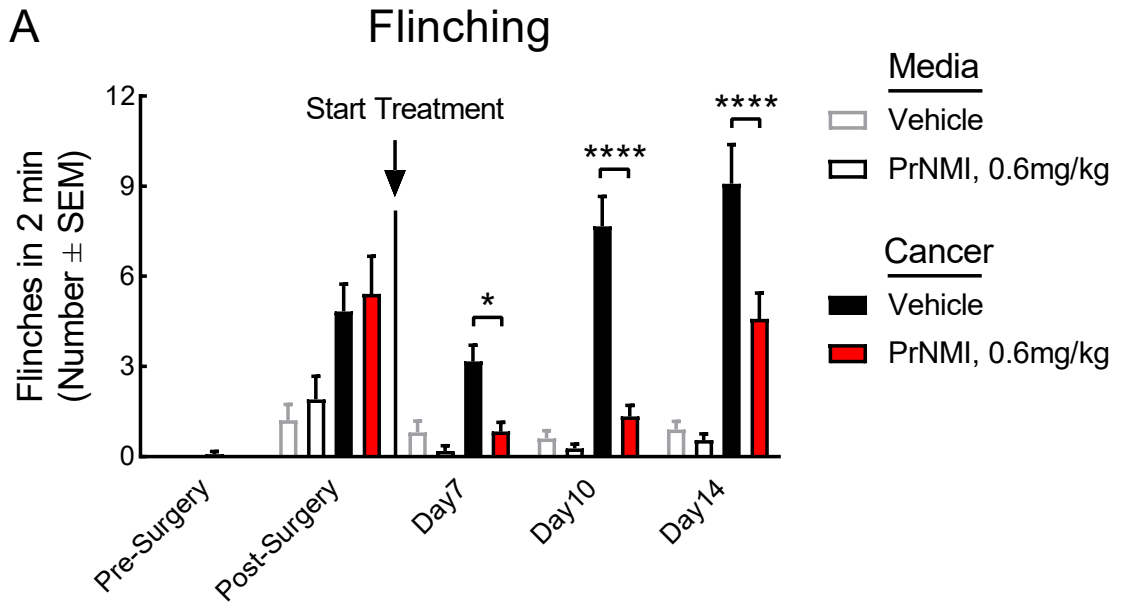


**Figure 5 Acute administration of PrNMI attenuates pain-induced spontaneous guarding in a cancer-induced pain model**

On day 7 after femoral inoculation with 66.1 breast cancer cells or cell-free media, animals were treated with PrNMI (0.1, 0.3, or 0.6 mg/kg, i.p.) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg, i.p.), and spontaneous pain behavior guarding was recorded in a 2-minute period at various time points. (A) A schematic diagram for spontaneous guarding in mice. (B) No significant difference was observed in spontaneous guarding between PrNMI-treated and vehicle-treated cancer-bearing mice. No significant difference was observed in media-only control animals between PrNMI-treated and vehicle-treated groups. Values represent the mean  $\pm$  SEM, n = 10-12 per group.

### **3.2 Sustained administration of PrNMI attenuates cancer-induced bone pain**

Next, we investigated whether sustained administration of PrNMI may boost the suppressing effect on pain-induced spontaneous guarding or induce tolerance in the animals. Cancer-bearing mice treated with vehicle presented with spontaneous flinching and guarding starting at day 7 and increasing through day 14 (Figure 6 A and B). Repeated administration of PrNMI (0.6 mg/kg, i.p., q.d., from day 7 to day 14) significantly attenuated both flinching and guarding on days 10 and 14 post-surgery when compared to vehicle-treated cancer-bearing mice (Figure 6 A and B).

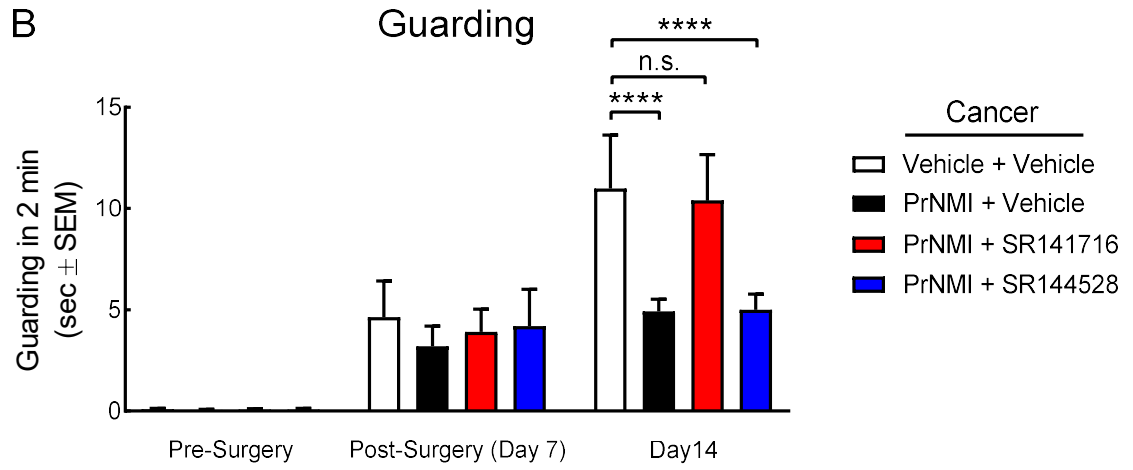
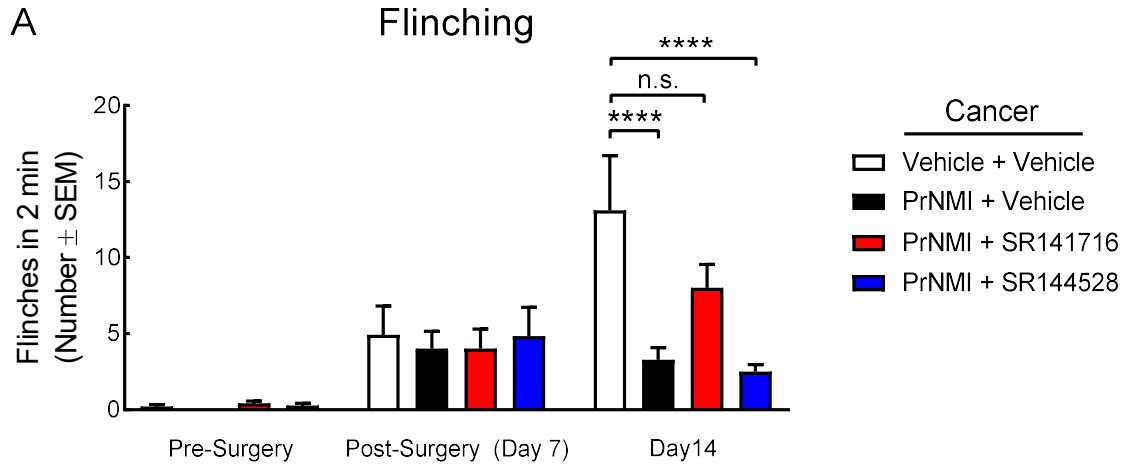


**Figure 6 Sustained administration of PrNMI attenuates spontaneous pain in a cancer-induced pain model**

On day 7 after femoral inoculation, animals demonstrated bone cancer-induced (A) flinching and (B) guarding. PrNMI (0.6 mg/kg, i.p.) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg, i.p.) was administered after behavioral measurements on day 7 and continued to day 14 (q.d.). Spontaneous flinching and guarding were recorded at 3-hour time points after treatment on days 7, 10 and 14. (A and B) Spontaneous flinching and guarding were significantly reduced by PrNMI compared to animals that received vehicle on day 10 and 14. For both flinching and guarding, no significant difference was observed in media-only control animals between PrNMI-treated and vehicle-treated groups. \* $p < 0.05$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ; values represent the mean  $\pm$  SEM,  $n = 8-12$  per group.

### **3.3 PrNMI attenuated cancer-induced bone pain by targeting CB1 receptors**

Although our previous study has demonstrated PrNMI as an effective CB1R agonist, it may also possess a weak agonist activity on CB2Rs [116]. To investigate its selectivity on CB1Rs, we treated the animals with selective CB1R or CB2R antagonists before the administration of PrNMI. The results showed that pre-treatment with selective CB1R antagonist SR141716 (1 mg/kg, i.p., q.d., from day 7 to day 14) suppressed the antinociceptive effect produced by PrNMI, while the administration of a selective CB2R antagonist SR144528 (1 mg/kg, i.p., q.d., from day 7 to day 14) did not (Figure 7 A and B). To confirm that the antinociceptive effect of PrNMI occurs by targeting peripheral cannabinoid receptors, we injected the antagonist (SR141716 or SR144528, 5  $\mu$ g / 5  $\mu$ L) or the vehicle spinally into the animals receiving chronic PrNMI. The results indicate no significant difference in pain behaviors was displayed between the antagonist and vehicle groups (Figure 8 A and B). Together, these data indicate that the administration of PrNMI attenuates CIBP by targeting peripheral CB1Rs.

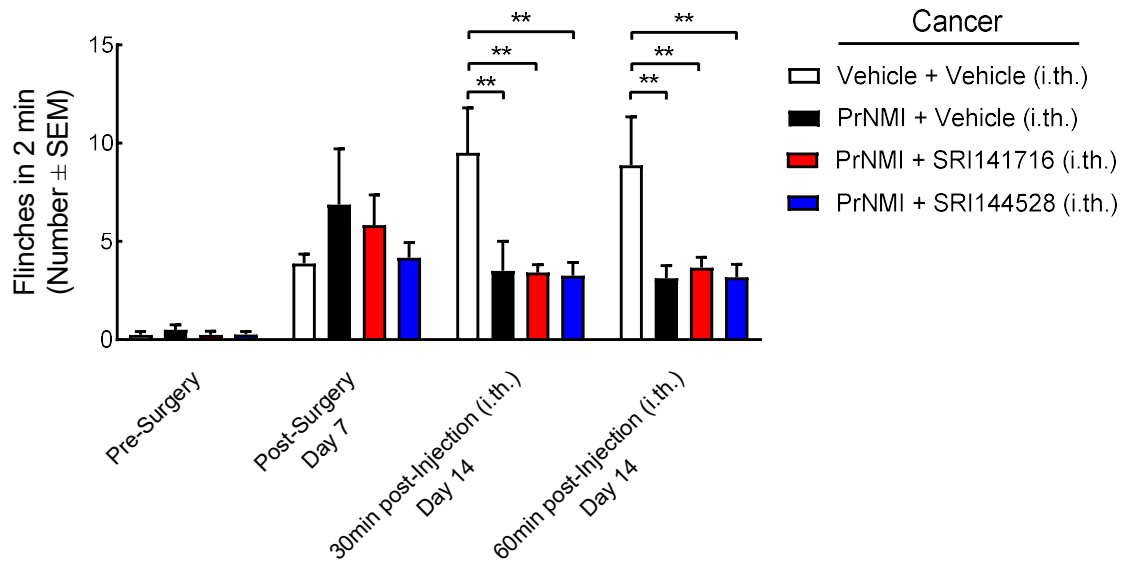


**Figure 7 Sustained administration of PrNMI attenuates spontaneous cancer-induced bone pain through the actions of CB1Rs**

On day 7 after femoral inoculation, animals demonstrated bone cancer-induced (A) flinching and (B) guarding. PrNMI (0.6 mg/kg, i.p.) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg, i.p.) was administered after behavioral measurements on day 7 and continued to day 14 (q.d.). Spontaneous flinching and guarding were recorded at 3-hour time points after treatment on days 7, 10 and 14. (A and B) The attenuation of bone cancer-induced flinching and guarding by PrNMI on day 14 was inhibited by pretreatment with the selective CB1R antagonist SR141716 (1 mg/kg, i.p., q.d., 10 minutes prior to PrNMI) but not inhibited by the selective CB2R antagonist SR144528 (1 mg/kg, i.p., q.d., 10 minutes prior to PrNMI). \*\*\*\* $p < 0.0001$ ; n.s., not significant; values represent the mean  $\pm$  SEM, n = 8-12 per group.

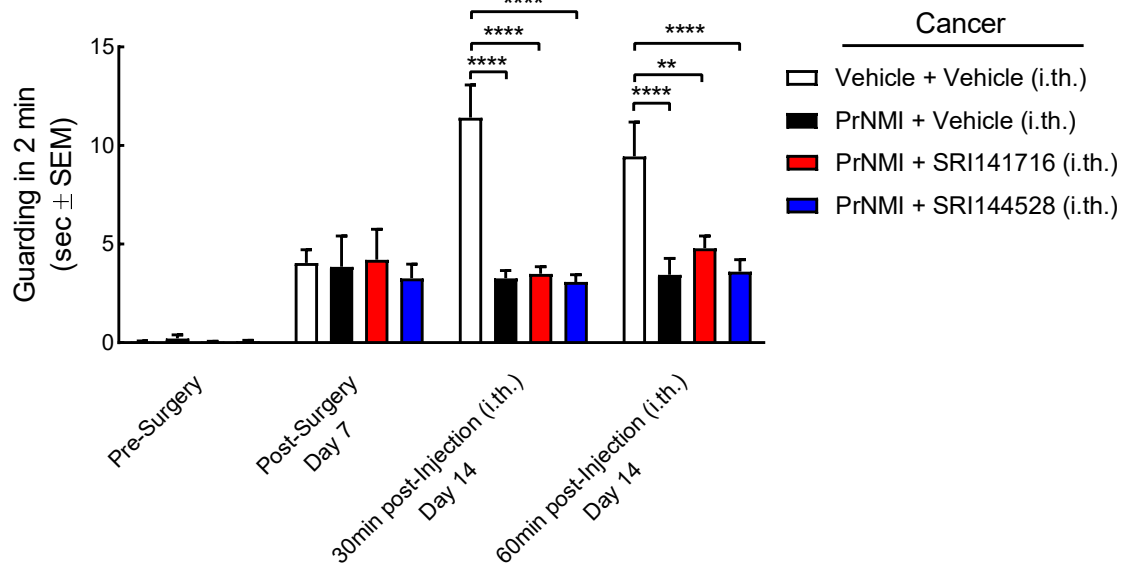
A

### Flinching



B

### Guarding



**Figure 8 Sustained administration of PrNMI attenuates spontaneous cancer-induced bone pain through the actions of peripheral CB1Rs**

On day 7 after femoral inoculation, animals demonstrated bone cancer-induced (A) flinching and (B) guarding. PrNMI (0.6 mg/kg, i.p.) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg, i.p.) was administered after behavioral measurements on day 7 and continued to day 14 (q.d.). Spontaneous flinching and guarding were recorded at 3-hour time points after treatment on days 7, 10 and 14. (A and B) Spinal administration of either SR141716 or SR144528 (5 µg per 5 µL, 2.5 hours post PrNMI treatment) did not inhibit the antinociceptive effect of PrNMI suggesting actions at peripheral receptors. \*\*p < 0.01, \*\*\*\*p < 0.0001; values represent the mean ± SEM, n = 8-12 per group.

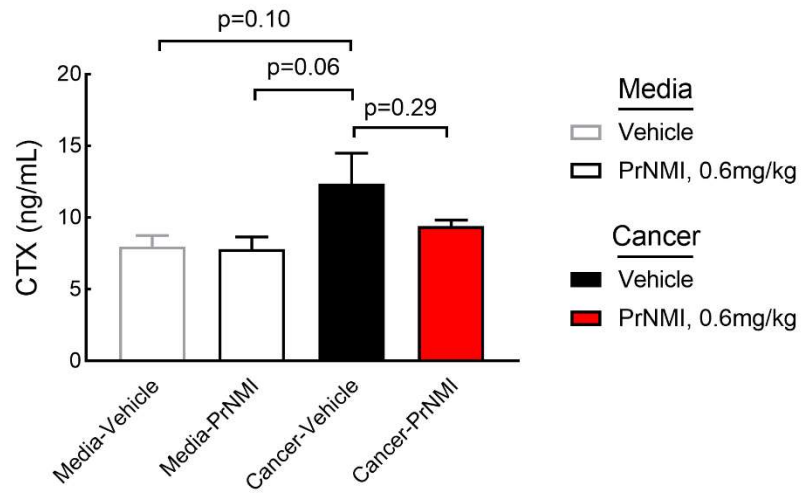
### **3.4 Sustained PrNMI does not alter bone integrity in cancer-bearing mice**

Different from other chronic pain conditions, the development of cancer-induced bone pain includes bone-specific mechanism. Therefore, the medications affecting bone remodeling may modulate the development of this pain state. Cannabinoids have been shown to regulate bone remodeling but whether their effects are beneficial or detrimental is still under debate [34,163]. To determine what is the effect of PrNMI on bone integrity, we investigated the bone remodeling in the cancer-bearing animals after chronic treatment of PrNMI. We first measured the serum levels of carboxy-terminal collagen crosslinks (CTX) in the animals, which was previously identified as a marker of bone resorption [164,165]. Our data showed that cancer-bearing animals had a higher level of CTX in their serum compared to media groups although no significant difference was observed (Figure 9 A). Interestingly, PrNMI seems to suppress the elevation of CTX in the cancer-bearing animals (Figure 9 A), suggesting PrNMI may inhibit cancer-induced bone degradation.

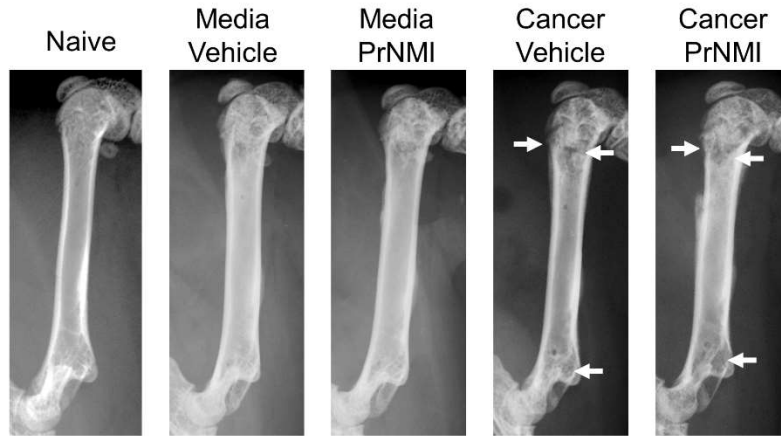
In addition to serum CTX, we also investigated the bone remodeling via bone radiographic imaging. According to the radiographic images taken on day 14 after cancer surgery, mice injected with media only displayed mild levels of bone loss primarily located at the distal femur head (Figure 9 B), while mice inoculated with cancer cells presented with severe levels of bone loss and even bone fracture (Figure 9 B). However, based on the radiographic images of these cancer-bearing animals, PrNMI did not seem to alter bone remodeling in mice either inoculated with cancer or media (Figure 9 B). To quantify the bone loss levels, we had three

blinded observers rated these radiographic images with an established scoring scale (0 = healthy bone, 1 = 1-3 lesions, 2 = 4-6 lesions, 3 = unicortical bone fracture, and 4 = bicortical bone fracture). Our results showed that mice injected with media only displayed a mild bone loss in the femurs (16 out of 20 mice have a score of 1 or less) (Figure 9 C). In contrast, almost all cancer-bearing mice experienced severe bone loss (21 out of 24 mice have a score of 2 or more) (Figure 9 C). Interestingly, different from what we found in serum CTX assay, sustained treatment of PrNMI (0.6 mg/kg, i.p., q.d., from day 7 to day 14 after surgery) did not alleviate or worsen cancer-induced bone degradation compared to cancer-vehicle group (Figure 9 C).

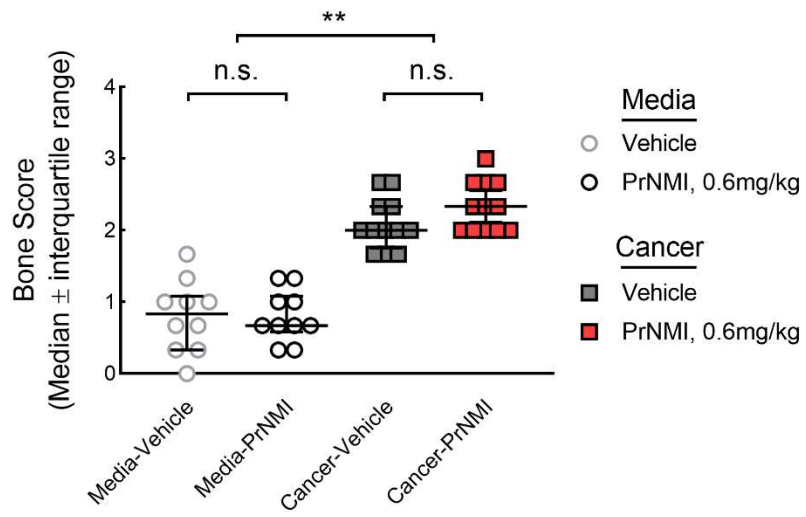
A



B



C

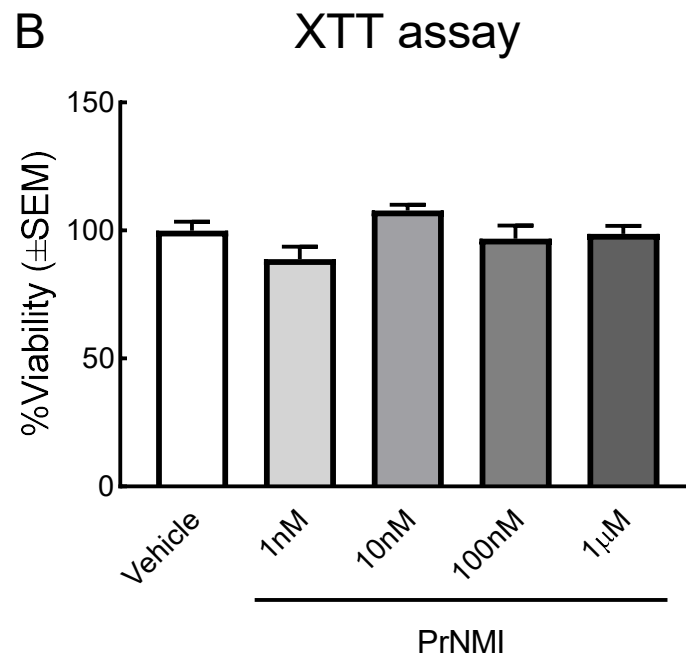
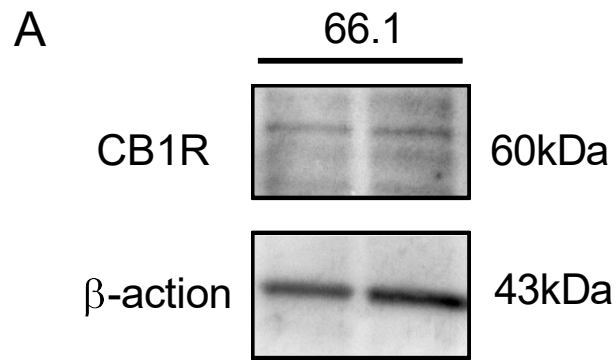


**Figure 9 Sustained administration of PrNMI does not exacerbate bone integrity**

Seven days after cancer or media inoculation, daily treatment of PrNMI (0.6 mg/kg, i.p.) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg, i.p.) was applied to all mice until day 14 post-surgery. On the last day of injection, radiographic images were taken, and sera were collected from these mice. (A) Levels of serum CTX were measured with a CTX ELISA kit. A possible elevation of serum CTX was observed in cancer-vehicle mice although no significant difference was observed compared to all other groups. (B) Representative radiographic images of femurs acquired from naïve, media- or cancer-inoculated mice. (C) Quantification of bone loss according to an established scoring scale (0 = healthy bone, 1 = 1-3 lesions, 2 = 4-6 lesions, 3 = unicortical bone fracture, and 4 = bicortical bone fracture). Cancer-bearing mice had more severe bone loss than sham mice. PrNMI treatment did not significantly change cancer induced bone loss. \*\*p < 0.01, n.s., not significant; values represent the median ± interquartile range, n = 6-9 per group (CTX assay), 10-12 per group (radiographic imaging).

### **3.5 PrNMI does not alter cancer cell viability in vitro**

In addition to bone remodeling, tumor growth is also a specific factor in the development of cancer-induced bone pain since tumor proliferation is directly associated with the exacerbation of pain and bone degradation in cancer patients [15]. Previous studies have shown that cannabinoids exert profound anti-tumor effects while opposite reports also exist [166]. In our study, the 66.1 murine breast cancer cells used to establish our cancer pain model were identified to express CB1Rs (Figure 10 A). Therefore, we would like to determine whether our peripherally restricted CB1R agonist PrNMI may affect tumor growth. In the study, 66.1 breast cancer cells were treated in vitro with varying concentrations of PrNMI (1 nM – 1  $\mu$ M) or vehicle for 24 hours, and the cell proliferation was then assessed with an XTT assay. Our results showed that none of the PrNMI treatments significantly changed cell viability compared to vehicle-treated cells (Figure 10 B), suggesting that PrNMI at the concentrations tested here does not alter cancer cell viability in vitro.



**Figure 10 PrNMI does not alter the viability of 66.1 breast tumor cells in vitro**

(A) CB1 receptors are expressed on 66.1 breast cancer cells. (B) 66.1 breast cancer cells do not have a change in their viability when treated with PrNMI. 66.1 breast cancer cells were plated into a 96-well plate at a density of  $1 \times 10^4$  per well. 24 hours later, cells were treated with different concentrations of PrNMI. After a 24-hour incubation, the cell viability was tested by using XTT assay. Values represent the mean  $\pm$  SEM, n = 12 per group.

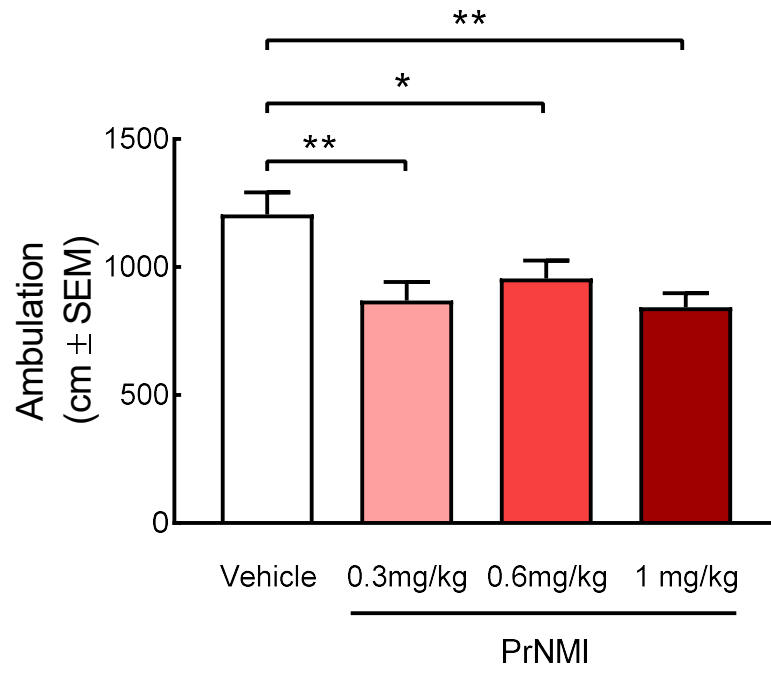
## **CHAPTER 4: SIDE EFFECTS PRODUCED BY THE PERIPHERALLY RESTRICTED CB1R AGONIST PRNMI**

### **4.1 Central side effects produced by PrNMI**

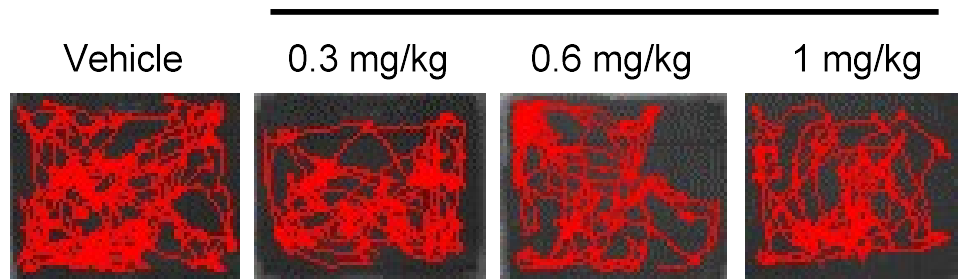
Previous studies suggested that the application of brain-permeant CB1R agonists can lead to central nervous system adverse consequences including sedation, motor incoordination, hypothermia and catalepsy [157,167], which largely limit the value of these cannabinoids as therapeutic reagents. Here we performed open field, rotarod, core temperature and ring immobility tests to determine whether PrNMI produces these adverse effects. In the open field test, a single administration of PrNMI (0.6 mg/kg, i.p.) significantly decreased the distance that mice traveled within a 5-minute period (Figure 11 A). However, the tracking patterns, center time and moving time performed by these naïve mice were not altered (Figure 11 B-D). In addition, PrNMI (0.6 mg/kg, i.p.) did not reduce the time that naïve mice spent on the rotating rod when compared to vehicle-treated mice (Figure 12). Rectal temperature in naïve mice was significantly decreased when treated with 1 mg/kg PrNMI compared to vehicle group, a dose 10-fold higher than the approximate ED<sub>50</sub> analgesic dose (0.1 mg/kg) (Figure 4 C and 13), yet mice treated with 0.6 mg/kg or lower doses of PrNMI did not show a significant decrease in their body temperature (Figure 13). PrNMI also produced a significant increase

in the time that mice spent motionless in the ring test when mice were treated with 0.6 mg/kg or a higher dose of PrNMI (Figure 14).

# A Distance Travelled

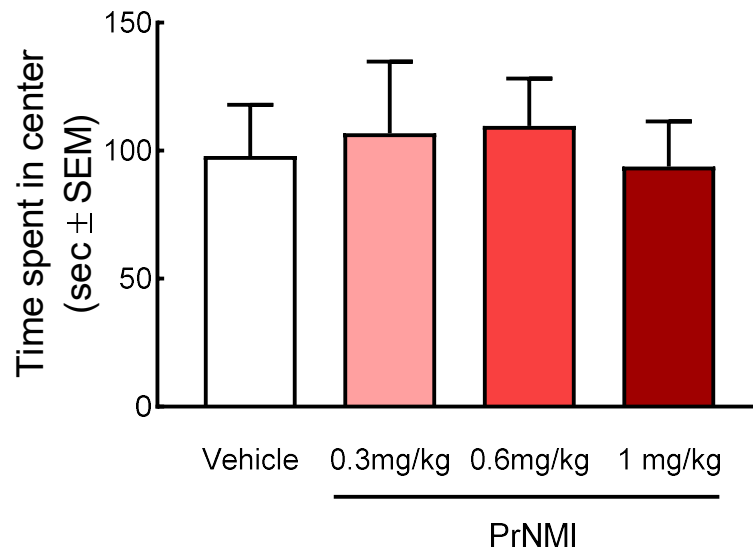


# B PrNMI



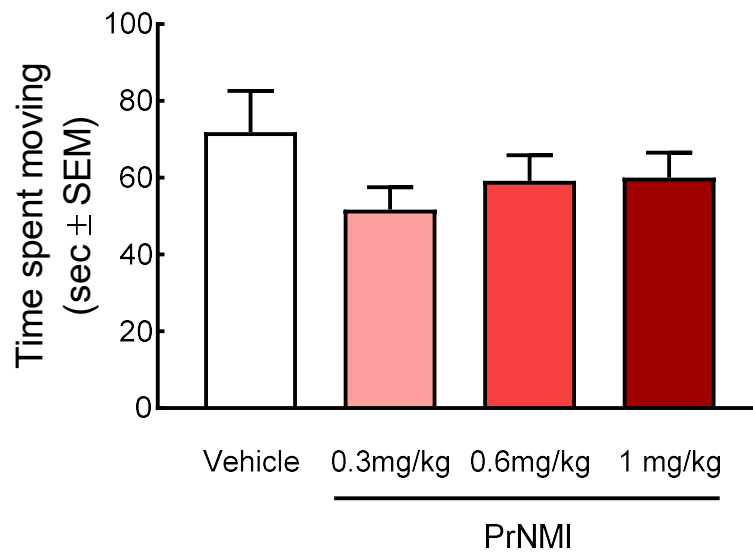
C

### Center Time



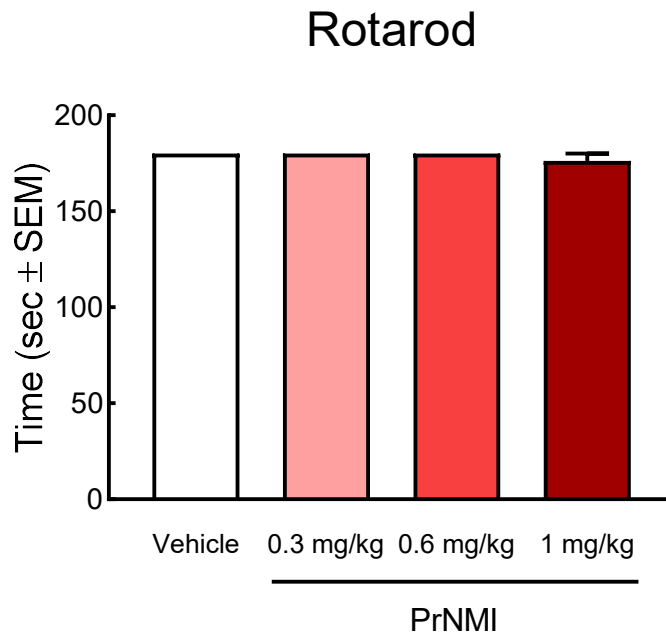
D

### Moving Time



**Figure 11 PrNMI administration does not produce anxiety nor a decrease in ambulation but results in a decrease in the distance traveled in the open field test**

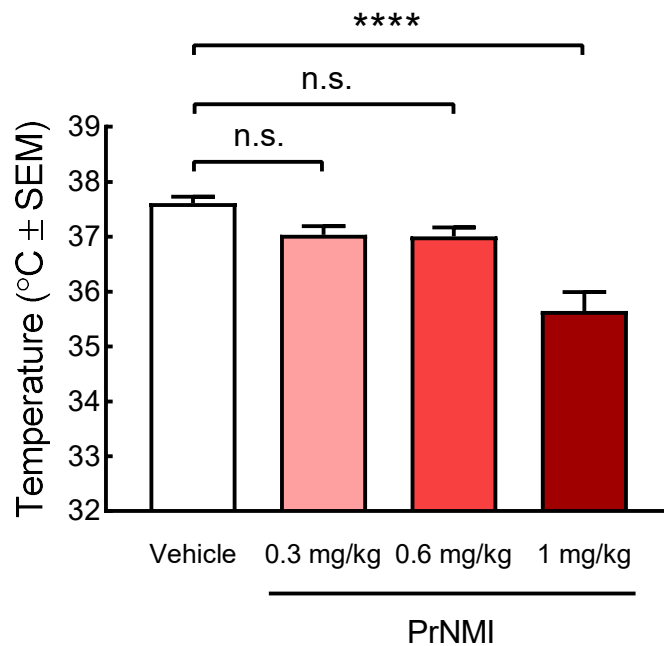
3 hours prior to open field test, mice were injected with either PrNMI (0.3, 0.6 or 1 mg/kg, i.p.) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg). (A) Mice given an acute administration of PrNMI did not show a significant difference in the (A) tracking patterns, (C) center time (the time the mouse stepped both front limbs in the center rectangle), and (D) moving time (the time the mouse moved at least one hind limb). However, significant difference was observed in the (B) travel distance between PrNMI-treated and vehicle-treated mice. \* $p < 0.05$ , \*\* $p < 0.01$ ; values represent the mean  $\pm$  SEM,  $n = 12$  per group.



**Figure 12 PrNMI does not impair motor function**

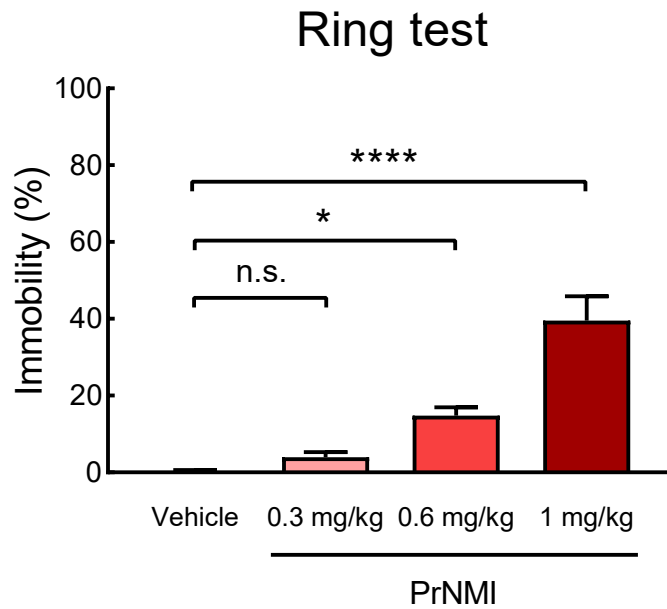
Mice were intraperitoneally injected with either PrNMI (0.3, 0.6 or 1 mg/kg) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg) 3 hours prior to the test, and no significant difference was observed between each two groups. Values represent the mean  $\pm$  SEM, n = 10-12 per group.

## Core Temperature Test



### **Figure 13 PrNMI administration at analgesic doses for CIBP does not impair hypothermia**

Mice were intraperitoneally injected with either PrNMI (0.3, 0.6 or 1 mg/kg) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg) 3 hours prior to the test. Hypothermia was induced by PrNMI at the highest dose tested (1mg/kg) but not at those therapeutic doses for CIBP (0.3 and 0.6 mg/kg). \*\*\*\* $p < 0.0001$ ; n.s., not significant; values represent the mean  $\pm$  SEM,  $n = 10-12$  per group.



**Figure 14 PrNMI administration at analgesic doses for CIBP induces catalepsy**

Mice were intraperitoneally injected with either PrNMI (0.3, 0.6 or 1 mg/kg) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg) 3 hours prior to the test. Catalepsy was induced when PrNMI was used at doses of 0.6 and 1 mg/kg. No catalepsy was identified at the dose of 0.3 mg/kg. \* $p < 0.05$ , \*\*\*\* $p < 0.0001$ , n.s., not significant; values represent the mean  $\pm$  SEM,  $n = 10-12$  per group.

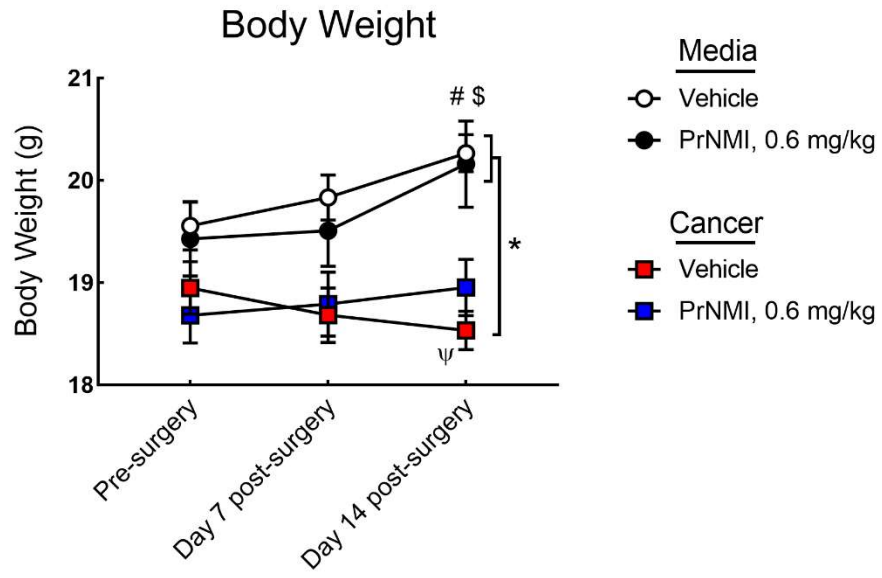
## 4.2 PrNMI promotes food intake in cancer-bearing mice

Hyperphagia is one of the side effects produced after cannabinoid consumption [168]. This effect was identified to be mediated via the CB1 receptors expressed in reward-associated brain regions, such as nucleus accumbens, lateral and paraventricular nuclei of the hypothalamus [168]. To determine if PrNMI may produce hyperphagia, we measured the body weight of the cancer-bearing mice after sustained PrNMI treatment. Our result showed that the body weight of cancer-bearing mice was significantly lower than media-treated mice on day 14 post-surgery (Figure 15 A). However, this result may be affected by the distinct initial body weight (day 0 before surgery) (Figure 15 A), so we also analyzed the changes of the body weight within each group between day 0 and day 14 post-surgery. The data indicate that mice from media groups (media-vehicle and media-PrNMI) had a significant increase in their body weight on day 14 (Figure 15 A). As no significant difference of the body weight was observed between these two groups on either day 0 or day 14, we conclude that PrNMI does not alter the food intake in non-cancer mice. In contrast, cancer-bearing mice (cancer-vehicle) had a significant reduction in their body weight on day 14 while no significant alteration of body weight was observed in cancer-bearing mice treated with sustained PrNMI (Figure 15 A). These data suggest that PrNMI may promote food consumption in cancer-bearing mice.

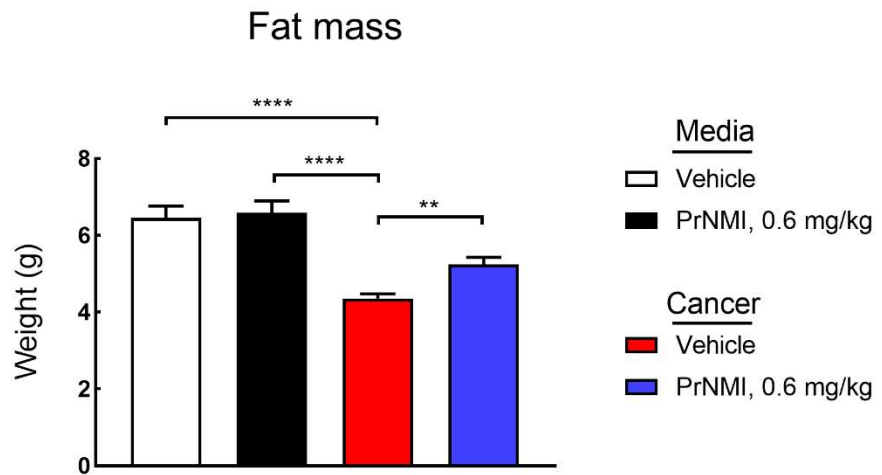
Additionally, we also measured the body composition, including fat mass and lean mass, in PrNMI-treated mice, which is another indicator of cannabinoid-induced hyperphagia. Our results found that cancer-bearing mice had a

significantly reduced fat mass in their bodies compared to mice in media groups on day 14 post-surgery (Figure 15 B). Sustained administration of PrNMI did not change the total fat mass in cancer-free mice but this treatment significantly increased fat weight in cancer-bearing mice (Figure 15 B), suggesting PrNMI specifically induces hyperphagic effect on mice with cancer. Lean mass is the total body weight without fat portion. Our results showed that no significant difference was found among all four groups of mice (Figure 15 C), implicating that PrNMI only affects fat mass but not other body compositions.

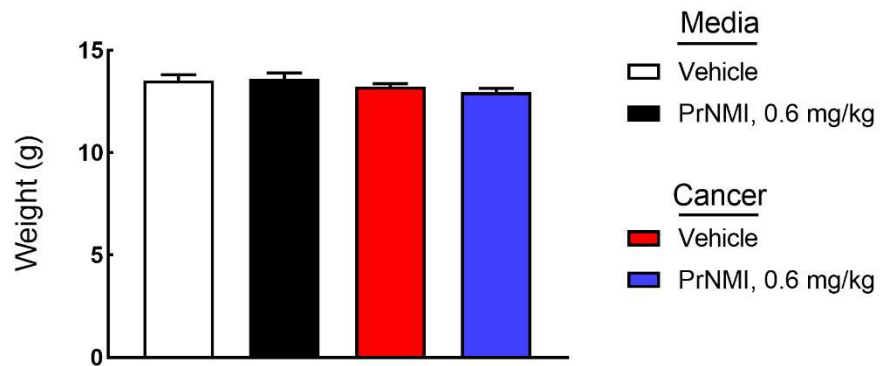
A



B



C



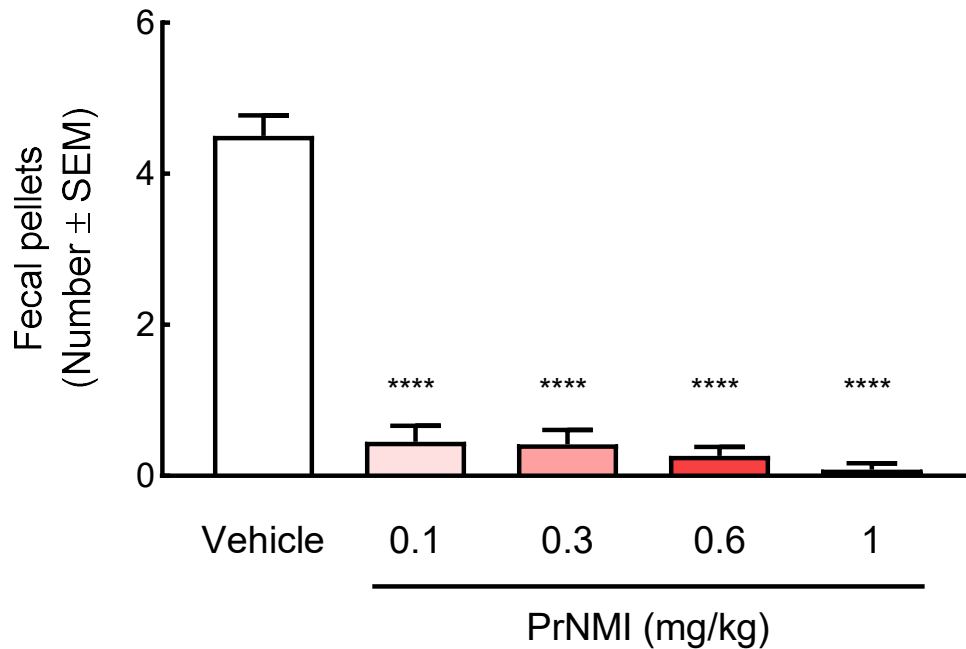
**Figure 15 Sustained administration of PrNMI promotes food intake in cancer-bearing mice but not in cancer-free mice**

In the experiments, mice received cancer surgery on day 0. Seven days later, daily treatment of PrNMI (0.6 mg/kg, i.p.) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg, i.p.) was applied to all mice until day 14 post-surgery. The body weight of all mice was measured on days 0, 7, and 14 post-surgery. The fat mass and lean mass were measured at the last day of injection. The alterations of body weight (A), fat mass (B) and lean mass (C) after sustained PrNMI or vehicle treatment in cancer- and media-inoculated mice. \* $p < 0.05$ , \*\*\*\* $p < 0.0001$ , significant difference between two comparing groups; # $p < 0.05$ , day 0 vs. day 14 post-surgery in media-vehicle group; \$ $p < 0.05$ , day 0 vs. day 14 post-surgery in media-PrNMI group;  $\psi p < 0.05$ , day 0 vs. day 14 post-surgery in cancer-media group. Values represent the median  $\pm$  interquartile range,  $n = 9-12$  per group (body weight),  $8-12$  per group (fat mass and lean mass).

### **4.3 PrNMI induces robust constipation in mice**

As we have discussed previously in the introduction, constipation is a severe problem in cancer patients who receive chronic opioid treatment. Unfortunately, this side effect is also observed after the use of cannabinoids. To determine if PrNMI also produces this side effect or not, we observed the ability of animals to defecate after injecting with different doses of PrNMI. According to our data, the number of fecal pellets produced by the animals were markedly reduced at all doses tested (Figure 16), suggesting PrNMI can induce constipation in mice.

## Defecation



### Figure 16 PrNMI produces robust constipation in mice

3 hours prior to the testing, mice were injected with either PrNMI (0.1, 0.3, 0.6 or 1 mg/kg, i.p.) or vehicle (80% saline, 10% DMSO, 10% Tween 80, 10 mL/kg). During the test, the mice were placed in an open arena for 5 min. The number of fecal pellets they produced within this period of time was recorded. PrNMI significantly suppressed the defecation ability of animals at all doses tested. \*\*\*\*p < 0.0001; values represent the mean ± SEM, n = 11-15 per group.

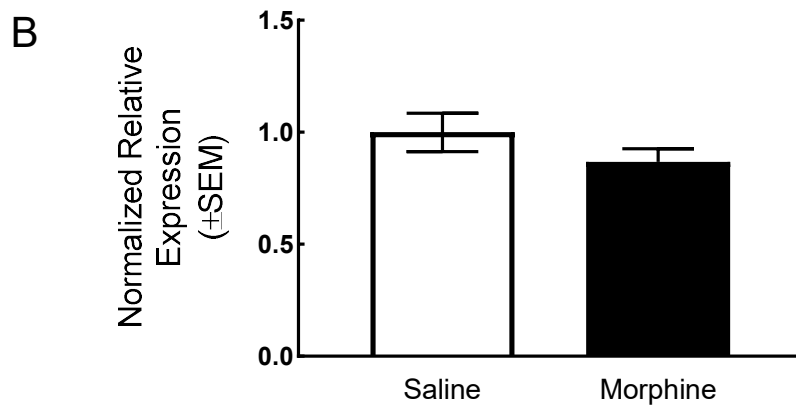
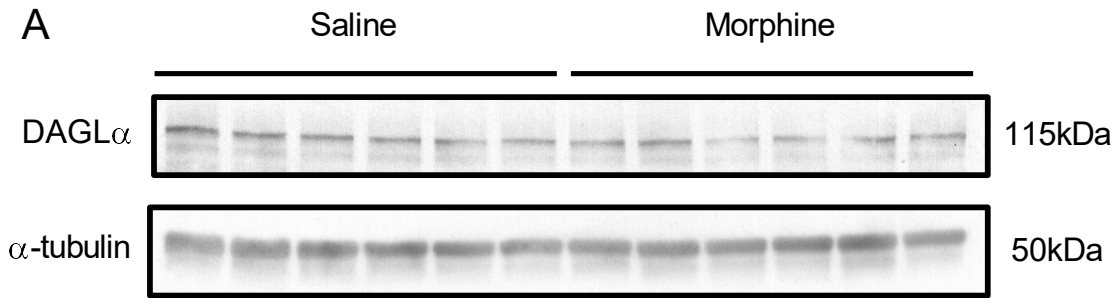
## **CHAPTER 5: THE EFFECTS OF CHRONIC MORPHINE ON THE ENDOCANNABINOID 2-AG AND CB2 RECEPTORS IN VTA**

### **5.1 The effects of chronic morphine on enzymes responsible for the synthesis and degradation of 2-AG**

As we mentioned previously, the production of 2-AG is regulated by multiple different enzymes. In the central nervous system, DAGL $\alpha$  and MAGL are the primary enzymes that directly control the synthesis and degradation of 2-AG respectively. Therefore, the changes in the expression and the activities of these two enzymes can significantly affect 2-AG production. Here we performed western blotting to determine if chronic morphine modulates the expression of these two proteins. The results showed that the expression of DAGL $\alpha$  was not altered after chronic morphine treatment (Figure 17). The MAGL expression trended towards being reduced by chronic morphine treatment, but no significant difference was found between the two treatment groups (Figure 18 A and B).

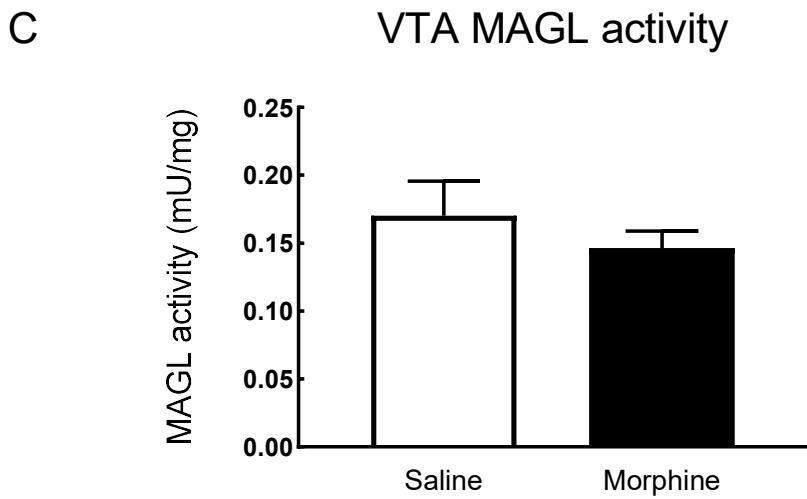
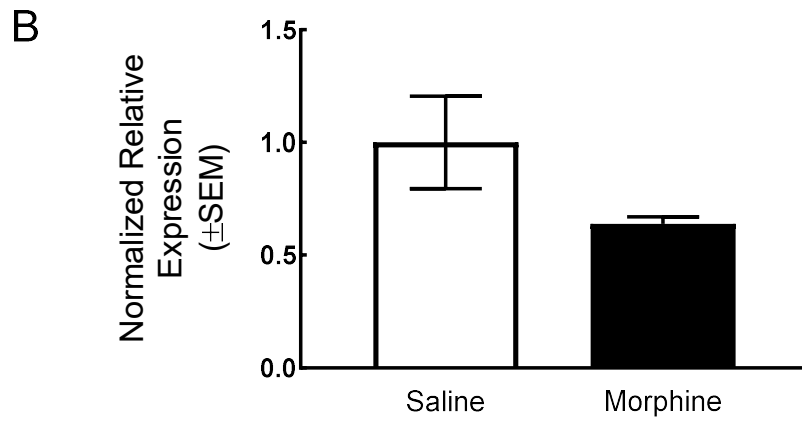
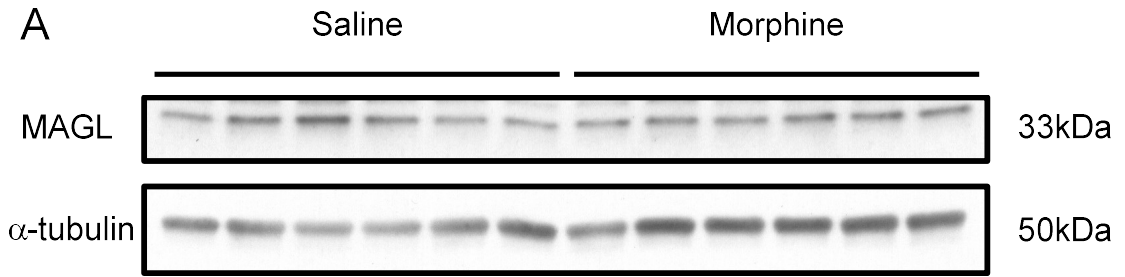
Previous studies showed that the regulation of MAGL occurs not only at its protein expression level, but could also at post-translational levels [169]. However, no antibodies are available for the examination of these post-translational modification to date. To investigate these possible changes on MAGL, we measured the overall activity of MAGL using a fluorometric activity assay (Figure 18 C). The results indicate that the VTA samples presented a total MAGL activity

of 0.15 and 0.17 mU/mL for chronic morphine and saline groups respectively while no significant difference was observed between two groups (Figure 18 C). Together, these data suggest that chronic morphine does not modulate the expression or the activity of DAGL $\alpha$  and MAGL.



**Figure 17 Chronic morphine does not alter the expression of DAGL $\alpha$  in VTA**

Rats were sacrificed after chronic morphine or saline treatment, and the VTA tissues were then collected and prepared for western blot analysis. (A) Samples were analyzed for the expression of DAGL $\alpha$ , the enzyme responsible for the synthesis of 2-AG. (B) Relative levels of DAGL $\alpha$  expression were determined by densitometric analysis and normalized to  $\alpha$ -tubulin in each lane. No significant difference in DAGL $\alpha$  expression was observed between two treatment groups. Values represent the mean  $\pm$  SEM, n = 6 per group.



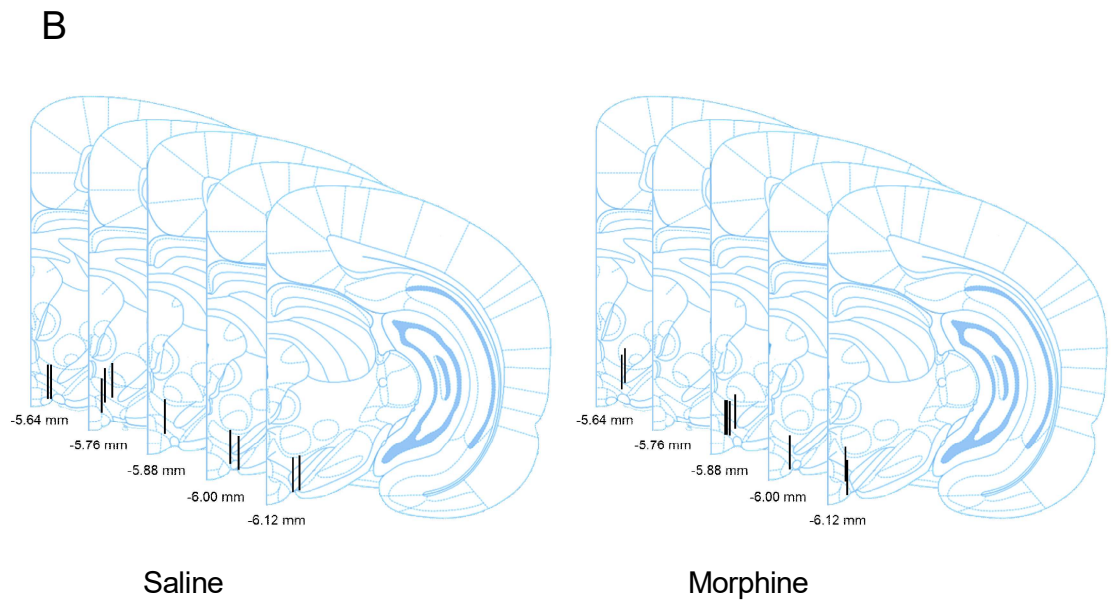
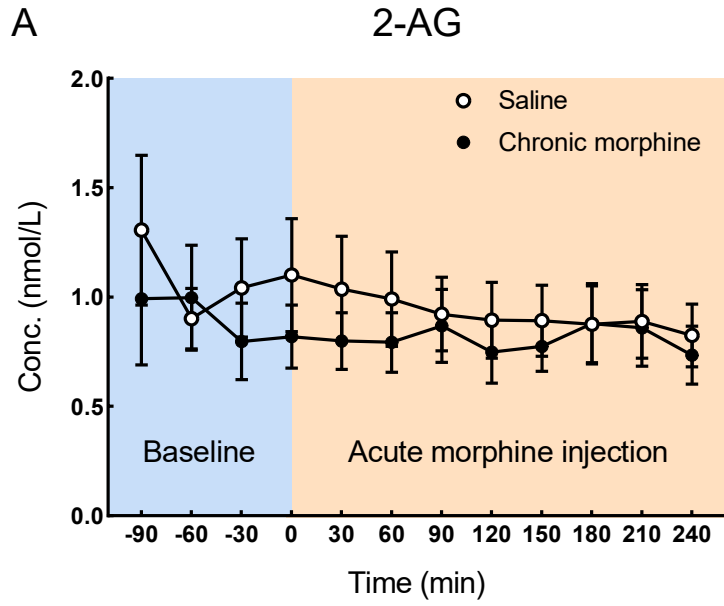
**Figure 18 Chronic morphine does not alter the expression or activity of MAGL in VTA**

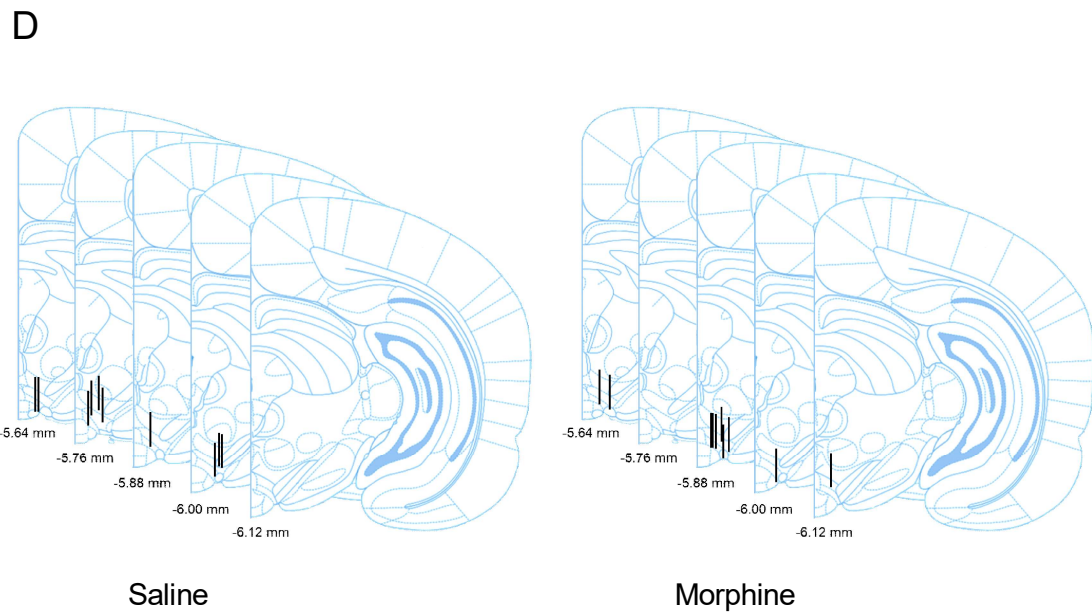
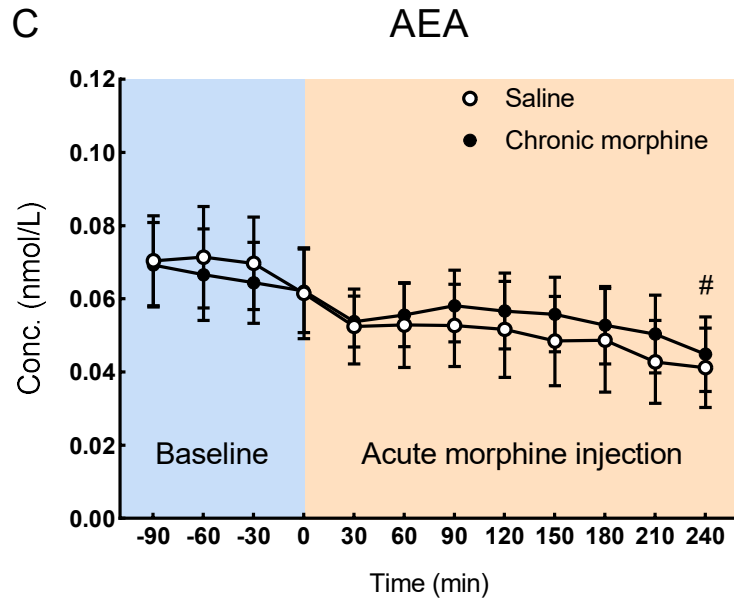
Rats were sacrificed after chronic morphine or saline treatment, and the VTA tissues were then collected and prepared for western blot analysis and MAGL activity assay. (A) Samples were analyzed for the expression of MAGL, the enzyme responsible for the degradation of 2-AG. (B) Relative levels of MAGL expression were determined by densitometric analysis and normalized to  $\alpha$ -tubulin in each lane. (C) The activity of MAGL in VTA samples was measured with a MAGL activity assay. No significant difference in MAGL expression or activity was observed between two treatment groups. Values represent the mean  $\pm$  SEM, n = 5-6 per group.

## 5.2 The effects of chronic morphine on the production of 2-AG

Although our data demonstrated that chronic morphine may not directly regulate the production of 2-AG via DAGL $\alpha$  and MAGL, it may still exert regulatory effects via a variety of other enzymes that are involved in the modulation of 2-AG production (See introduction). Therefore, to determine the actual influence of chronic morphine on the production of 2-AG, we employed in vivo microdialysis in the VTA of awake rats. Our results found that, one day after the chronic morphine injection, 2-AG production in VTA was not significantly altered compared to saline-treated group (Figure 19 A). However, considering the possibility that the chronic morphine-induced 2-AG alteration have returned to the baseline level, we administered an acute injection of morphine (5 mg/kg, i.p.) to both chronic morphine-treated and saline-treated rats and examined whether the 2-AG production may be altered compared to the baseline level. Again, we did not see significant difference of 2-AG production between two treatment groups or compared to baseline levels (Figure 19 A). In addition to 2-AG, we also examined the effects of chronic morphine on AEA production as a control. Similarly, no significant difference in AEA production was observed between chronic morphine-treated and saline-treated rats before (baseline session) or after acute morphine injection (morphine injection session) (Figure 19 C). However, we did identify a significant difference in AEA production between the baseline (t -90 to 0 min) and the last time point of the morphine injection session (t 240 min) (Figure 19 C), suggesting acute morphine but not chronic morphine may reduce the production

of AEA in VTA. The placement of all microdialysis guide cannulas was verified after experiments (Figure 19 B and D).





**Figure 19 Chronic morphine does not alter the production of endocannabinoids in VTA**

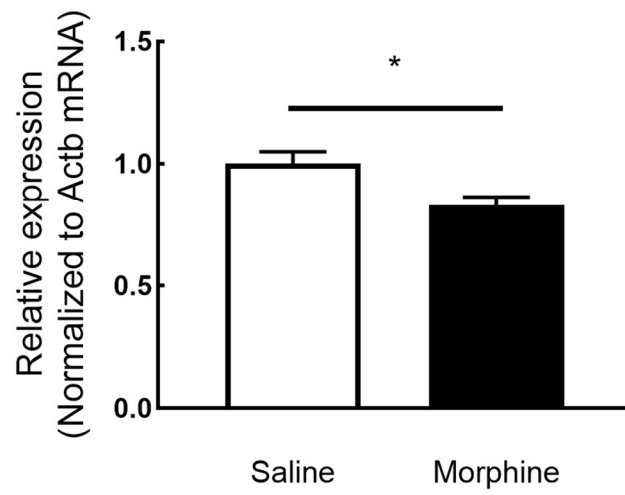
In vivo microdialysis was performed on rats one day after chronic morphine treatment to determine the alterations of endocannabinoids in VTA. Microdialysis samples were collected every 30 min for a total of 6 hours. After the first 2-hour baseline, all rats received an acute injection of morphine and the changes in the production of endocannabinoids was observed for the next 4 hours. (A and C) no significant difference in the production of either 2-AG or AEA was observed between treatment groups at baseline session or in the acute morphine session. However, a significant time effect in AEA production was observed between the baseline period and the last time point of the acute morphine session (t 240 min). (B and D) Anatomical representatives of microdialysis guide cannula placements in VTA for the studies of 2-AG and AEA. #p < 0.05, t 240 min vs. baseline (t -90 to 0 min). Values represent the mean  $\pm$  SEM, n = 9-10 per group.

### **5.3 The effects of chronic morphine on CB2 receptors**

Next, we investigated the effects of chronic morphine on CB2Rs. Due to the lack of specificity of current CB2R antibodies [170,171], we employed qRT-PCR to examine the mRNA expression of CB2Rs in VTA. Our results found that chronic morphine significantly decreased the mRNA expression level of CB2Rs by 17% (Figure 20 A). We also examined CB1R expression as a control by using both qRT-PCR and western blotting. The results showed no significant difference in the expression of CB1Rs between chronic morphine-treated and saline-treated groups (figure 20 B-D).

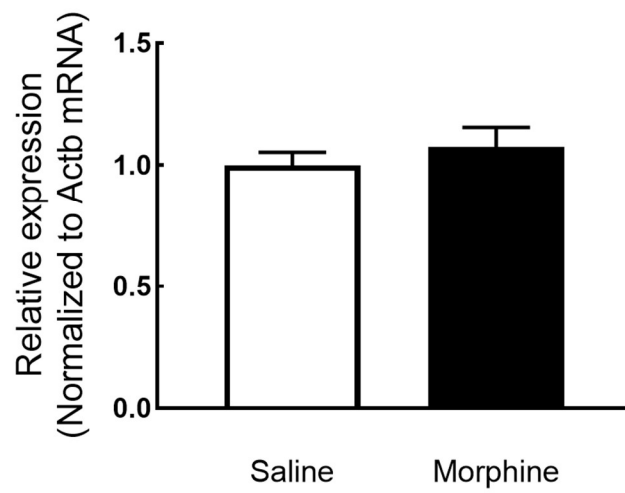
A

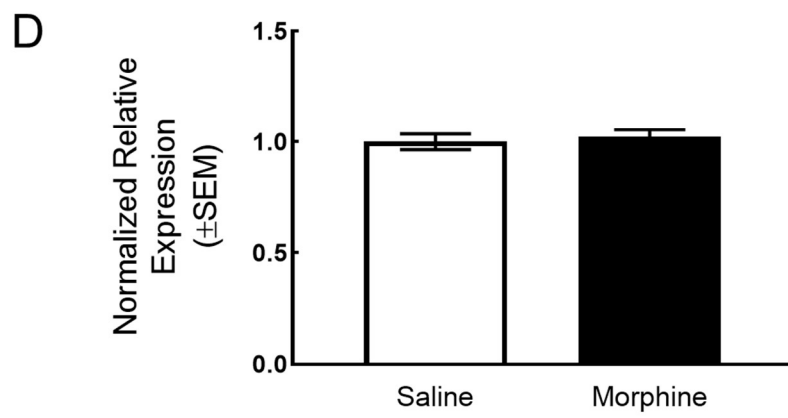
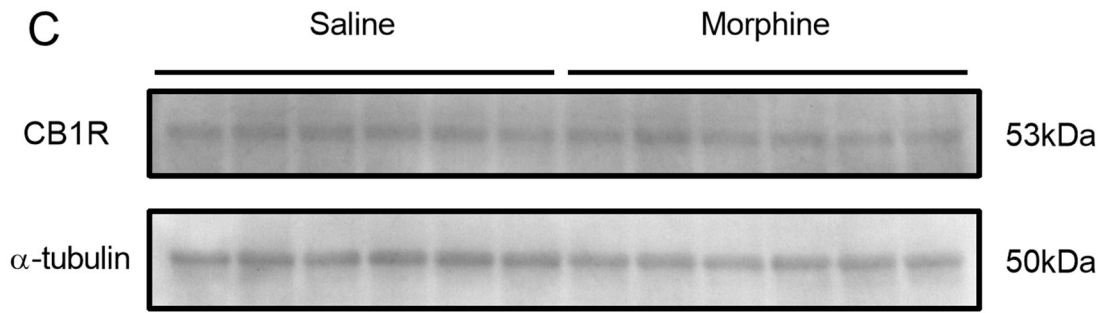
### VTA CB2R mRNA



B

### VTA CB1R mRNA





**Figure 20 Chronic morphine decreases the expression of CB2Rs but not CB1Rs in VTA**

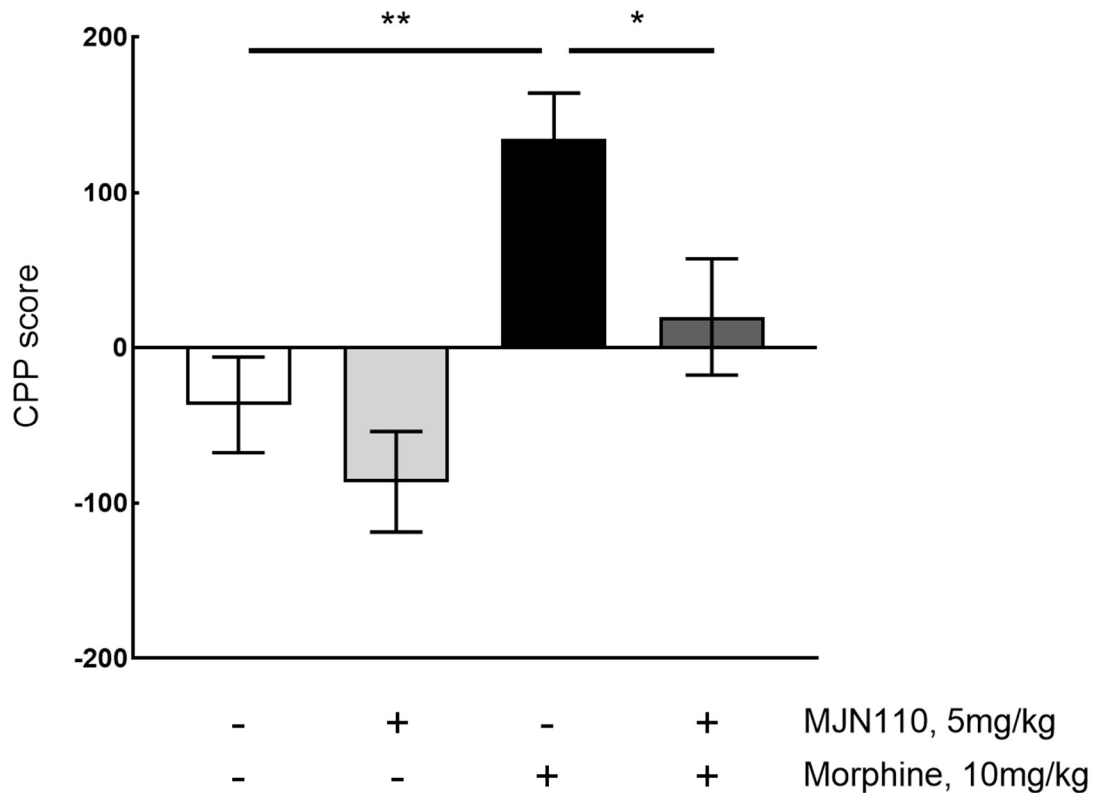
Rats were sacrificed after chronic morphine or saline treatment, and the VTA tissues were then collected and prepared for qRT-PCR and western blot analysis. Relative mRNA expression levels of (A) CB2Rs and (B) CB1Rs were determined by qRT-PCR and normalized to  $\beta$ -actin mRNA level. (C) Samples were analyzed for the protein expression of CB1Rs. (D) Relative protein expression of CB1R was determined by densitometric analysis and normalized to  $\alpha$ -tubulin in each lane. CB2R mRNA expression was significantly decreased in chronic morphine treatment, but no significant difference in CB1 expression at either protein level or mRNA level was observed between two treatment groups. \* $p < 0.05$ , morphine vs. saline. Values represent the mean  $\pm$  SEM,  $n = 6$  per group.

## **CHAPTER 6: THE ROLES OF ENDOCANNABINOID 2-AG AND CB2 RECEPTORS IN CHRONIC MORPHINE-INDUCED REWARD**

### **6.1 The modulatory effect of 2-AG on chronic morphine-induced reward**

Currently, no study has investigated the modulatory effect of 2-AG on chronic morphine-induced reward. To examine this, we performed the conditioned place preference test. According to our results, rats that received chronic morphine showed a significant preference to the drug-paired chamber compared to the saline-treated rats (Figure 21). The increase of 2-AG tone by pretreatment of a selective MAGL inhibitor, MJN110 (5 mg/kg, i.p.), markedly suppressed morphine-induced preference, indicating an inhibitory role of 2-AG in chronic morphine-induced reward (Figure 21). Interestingly, we observed that the rats received MJN110 but not morphine showed a trend of aversion to the drug-paired chamber although no significant difference was observed compared to vehicle-saline group (Figure 21). This suggests that the inhibitory effect of 2-AG on morphine-induced reward may be mediated via those brain regions associated with aversion, such as amygdala, anterior insula and anterior cingulate cortex [172].

## Conditioned Place Preference



**Figure 21 MJN110 attenuates chronic morphine-induced reward**

After baseline testing, rats were conditioned for 5 days in three-chamber CPP boxes using MJN110 (5 mg/kg, i.p.) / vehicle and morphine (10 mg/kg, i.p.) / saline. Their chamber preference was tested on the next day after conditioning. Rats that received chronic morphine presented a strong preference towards the drug-paired chamber but not saline-treated rats. MJN110 significantly suppressed morphine-induced preference. MJN110 may produce aversive effect on rats but no statistic difference was observed compared to the results from vehicle-saline-treated rats. \* $p < 0.05$ , \*\* $p < 0.01$ . Values represent the mean  $\pm$  SEM,  $n = 11-14$  per group.

## 6.2 The modulatory effect of CB2 receptors on chronic morphine-induced reward

The CB2 receptor is another element of the endogenous cannabinoid system that has not been investigated for its role in the modulation of chronic morphine-induced reward. Like 2-AG, we performed conditioned place preference to examine the modulatory effect of CB2R. Rats were pre-treated with a selective CB2R agonist, JWH015 (3 mg/kg, i.p.), or vehicle followed by morphine or saline treatment. The results showed that the activation of CB2Rs markedly inhibited morphine-induced preference (Figure 22), indicating an inhibitory role of CB2Rs in chronic morphine-induced reward. Different from MJN110, JWH015 treatment in rats without morphine injection did not present preference to any side chambers, demonstrating that CB2R activation does not produce trends towards an aversive effect (Figure 22).

Next, we wanted to investigate if CB2Rs located in VTA mediate the inhibitory effect on chronic morphine-induced rewarding behavior. To explore this, we knocked down the expression of CB2Rs using CRISPR/Cas9, a genomic editing technique (Figure 23 A). The single guide RNA (sgRNA) that specifically targets the CB2R gene was designed to match the 5' end of the first protein-coding exon included in both of the protein-coding transcripts of *cnr2* gene according to the Ensembl genome database (<http://www.ensembl.org>) (Figure 23 B). To verify the efficacy of the CRISPR/Cas9 gene editing mediated via our *cnr2*-sgRNA, we developed a target plasmid expressing the rat CB2R fused with a GFP tag (Figure 23 C). When this target plasmid is co-transfected with the *cnr2*-sgRNA/CRISPR

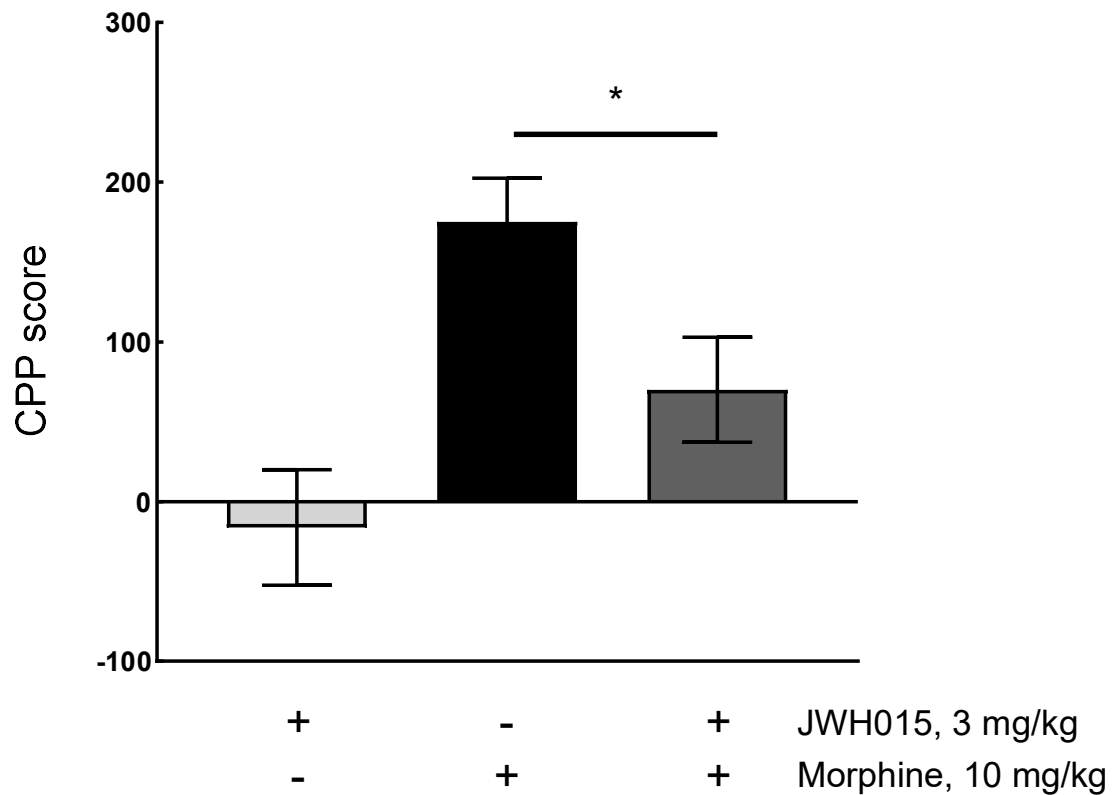
plasmid into mouse neuron-derived CAD cells, its ability to express CB2R-GFP fusion protein should be impaired if the rat *cnr2* gene has been effectively edited (Figure 23 C). Our results showed that the rat CB2R-GFP fusion protein was highly expressed in the cells when co-transfected with a control CRISPR plasmid (Figure 23 D and E). However, its expression has been markedly decreased when the *cnr2*-sgRNA-expressing CRISPR plasmid was transfected, demonstrating the *cnr2*-sgRNA can effectively mediate CRISPR editing of the rat *cnr2* gene (Figure 23 D and E). We directly microinjected this CRISPR/Cas9 plasmid in rat VTA to knock down the expression of CB2Rs (Figure 24 A). The animals were then tested using conditioned place preference two weeks after the CRISPR injection. Our data showed that, unexpectedly, the VTA injection of CB2R-CRISPR plasmid did not alter the chronic morphine-induced preference (Figure 24 B). This result indicates that the VTA CB2Rs may not modulate chronic morphine-induced reward or at least be involved in the development of chronic morphine-induced reward.

Nevertheless, there is still another possibility to explain the above results – the CRISPR system did not successfully knock down CB2Rs in VTA. Again, due to the lack of specific CB2R antibody, we can not validate the efficacy of CRISPR system in vivo using antibody-based techniques, such as western blotting and immunohistochemistry. In addition, as the mutations generated by CRISPR only affect CB2R expression at protein level (due to the shift of open reading frame) but not at mRNA level. The techniques that quantify the mRNA expression, such as qRT-PCR and RNA in situ hybridization are not useful in quantitating knockdown as well. To solve this conundrum, we tried a novel technique that was published

recently named TIDE (Tracking of Indels by DEcomposition) [150]. This technique can decompose the sequence trace data (DNA chromatogram) and allows us to have a comprehensive profile of all insertions and deletions (indels) in the CRISPR-edited samples as well as the editing efficiency of CRISPR (Figure 25 A) [150]. With this technique, we analyzed the genomic DNA samples extracted from our CRISPR-edited VTA tissues. Our data showed that rats microinjected with either CB2R-CRISPR or control-CRISPR only displayed an editing efficiency of less than 2% (Figure 25 B-F). These results indicate that the injection of CRISPR plasmid may not efficiently edit CB2R gene in VTA.

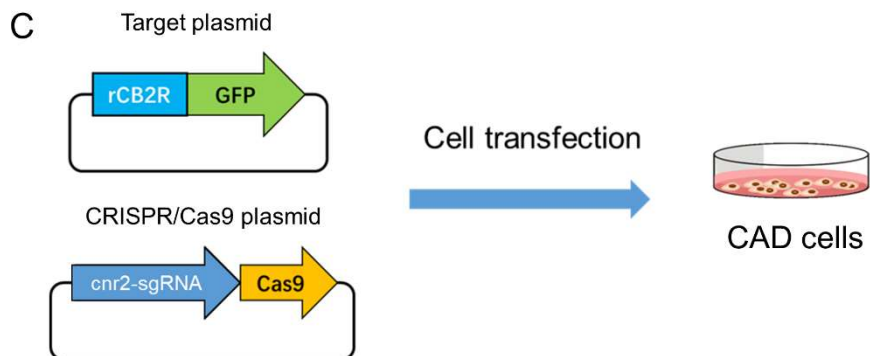
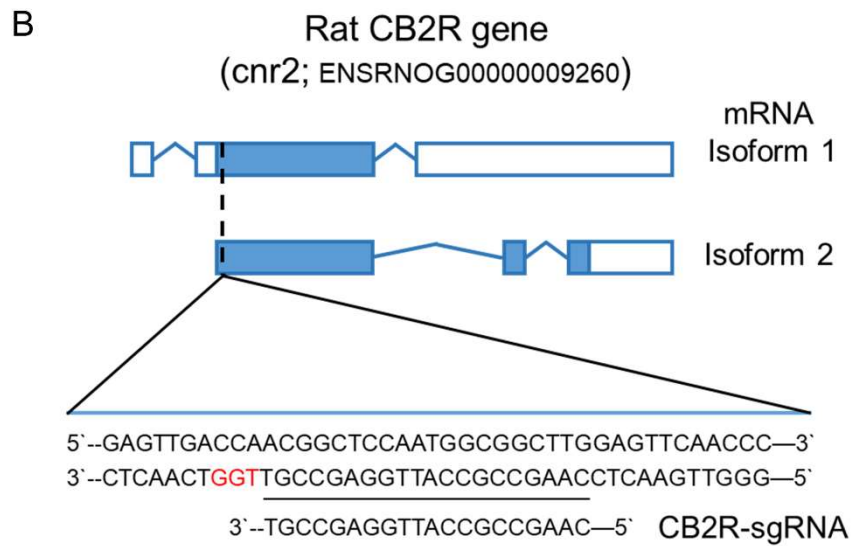
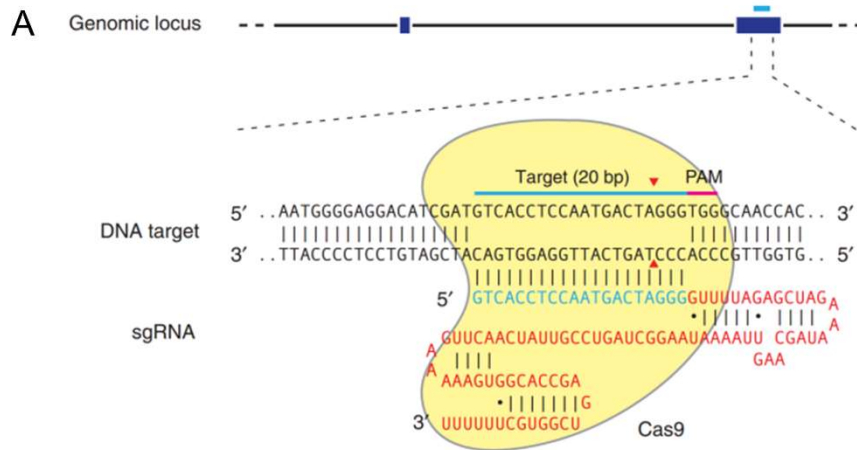
One possible reason for this low CRISPR editing efficiency is the low transfection efficiency of plasmid injection. To examine this possibility, we packaged our *cnr2*-sgRNA/CRISPR system into lentivirus and bilaterally microinjected into the rat VTA. The CRISPR editing efficiency was investigated by TIDE analysis. Our results again showed that the editing efficiency of CRISPR is very low, implicating that CRISPR may not efficiently edit CB2R gene in VTA (Figure 26 A-C).

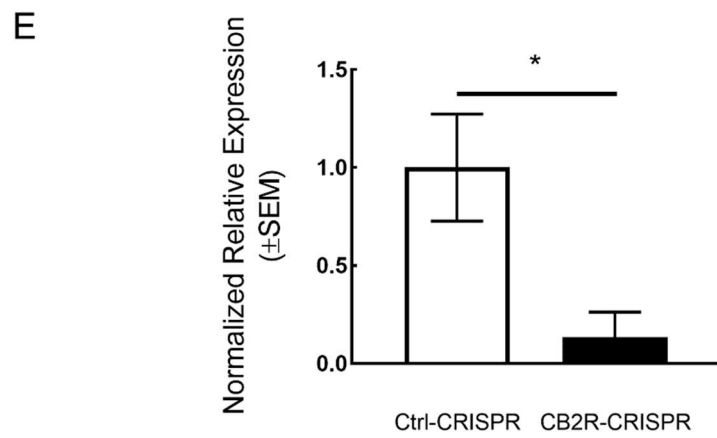
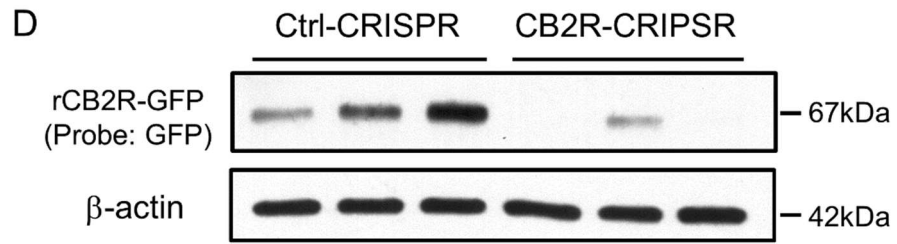
## Conditioned Place Preference



**Figure 22 JWH015 attenuates chronic morphine-induced reward**

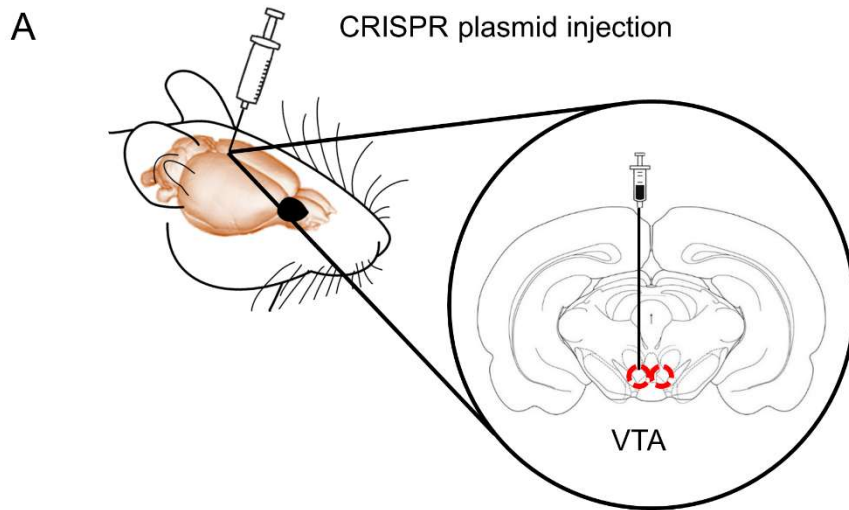
After baseline testing, rats were conditioned for 5 days in three-chamber CPP boxes using JWH015 (3 mg/kg, i.p.) / vehicle and morphine (10 mg/kg, i.p.) / saline. Their chamber preference was tested on the next day after conditioning. JWH015 attenuated morphine-induced preference but did not alter the preference in saline-treated rats. \* $p < 0.05$ . Values represent the mean  $\pm$  SEM,  $n = 11-16$  per group.





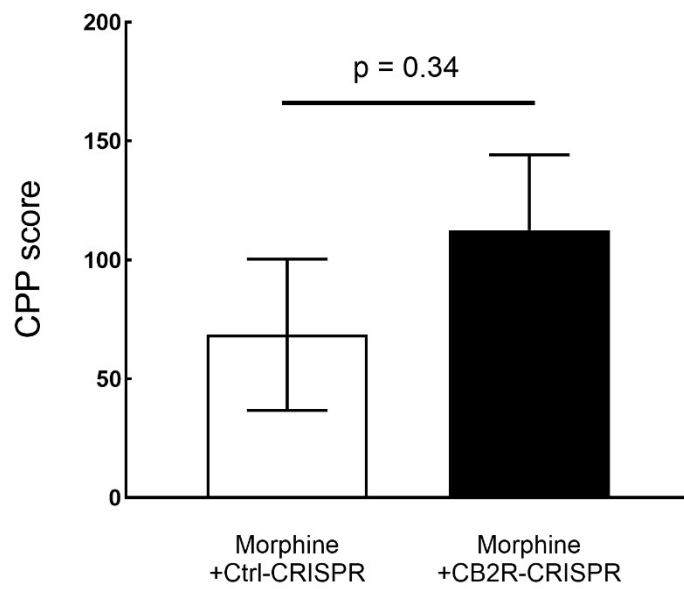
**Figure 23 *cnr2*-sgRNA/CRISPR system effectively knocked down rat CB2R expression in vitro**

(A) Schematic diagram of the sgRNA-guided CRISPR/Cas9 system. This figure was originally published in: Ran FA, et al. Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*, 2013, 8(11): 2281.[148] (B) Schematic diagram of rat CB2R gene transcripts and *cnr2*-sgRNA. Two protein-coding transcripts can be transcribed from rat CB2R gene, both of which include the first protein-coding exon. Our *cnr2*-sgRNA was designed to target the 5'-end of this exon in order to deactivate the function of the whole gene. (C) The editing efficiency of the *cnr2*-sgRNA/CRISPR system was verified using a rat CB2R-expressing target plasmid. When this target plasmid was co-transfected with CRISPR/Cas9 plasmid containing *cnr2*-sgRNA into CAD cells, the CB2R gene on the target plasmid was edited and lost the ability to express CB2R protein and GFP tag. (D) Protein samples acquired from transfected CAD cells were analyzed for the expression of CB2R-GFP fusion protein (Probed by GFP antibody). (E) Relative levels of GFP expression were determined by densitometric analysis and normalized to  $\beta$ -actin in each lane. The expression of CB2R-GFP fusion protein was significantly reduced in cells transfected with CB2R-CRISPR plasmid. \* $p < 0.05$ . Values represent the mean  $\pm$  SEM,  $n = 3$  per group.



B

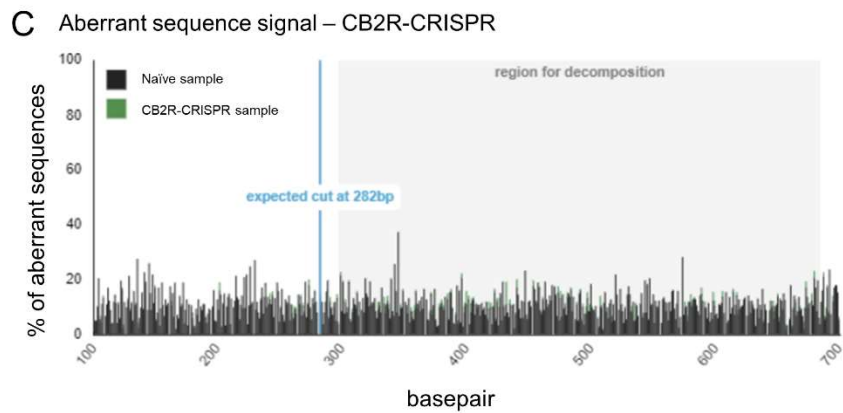
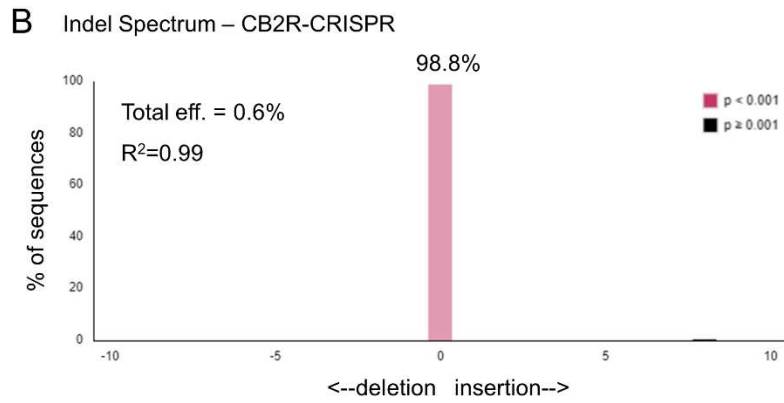
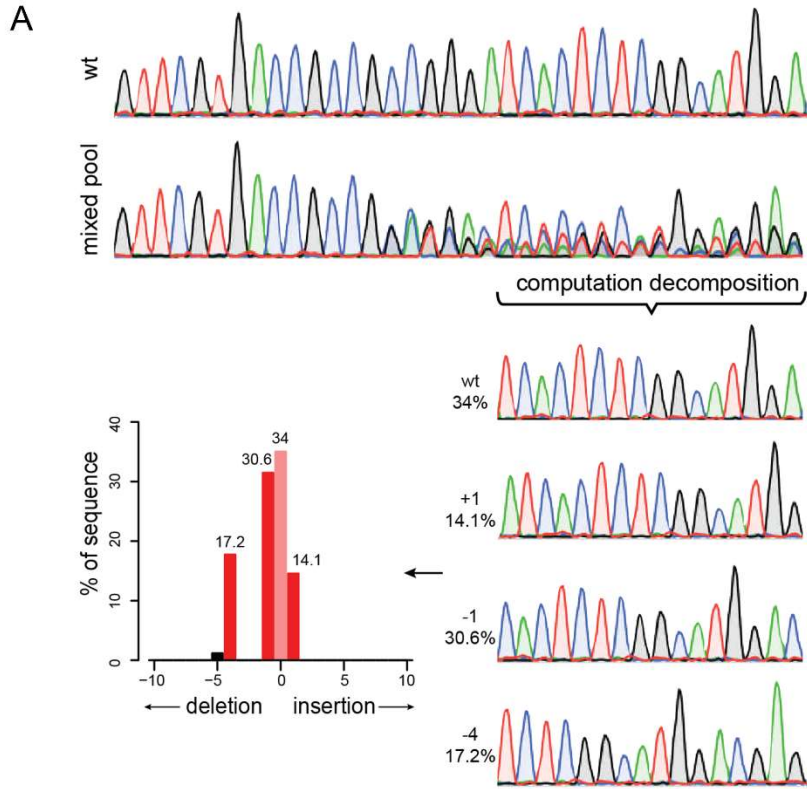
Conditioned Place Preference



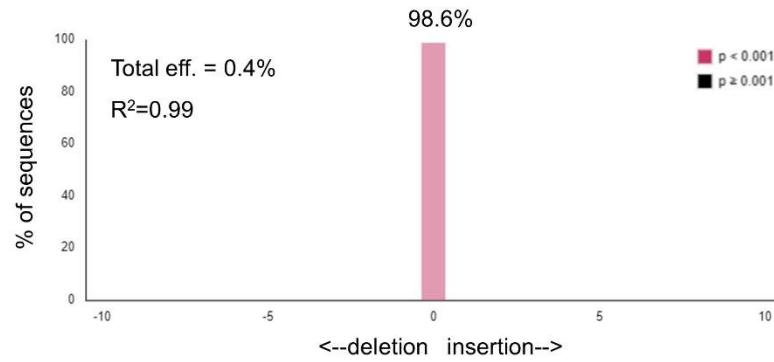
**Figure 24 Intra-VTA injection of CB2R-CRISPR did not alter chronic morphine-induced reward**

(A) Schematic diagram of intra-VTA injection of CB2R-CRISPR plasmid bilaterally.

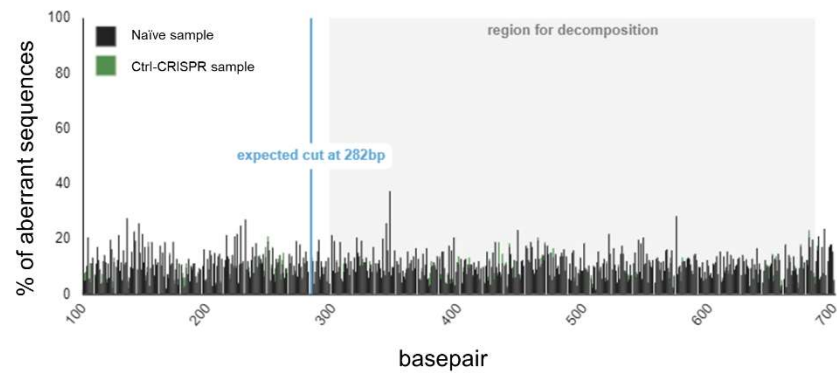
(B) After CRISPR injection, rats were allowed to recover for two weeks prior to CPP baseline testing. All rats were then conditioned for 5 days in three-chamber CPP boxes after morphine (10 mg/kg, i.p.) or saline treatments. Their chamber preference was tested on the next day after conditioning. Intra-VTA injection of CB2R-CRISPR did not attenuate morphine-induced preference. Values represent the mean  $\pm$  SEM, n = 10-12 per group.



**D** Indel Spectrum – Ctrl-CRISPR



**E** Aberrant sequence signal – Ctrl-CRISPR



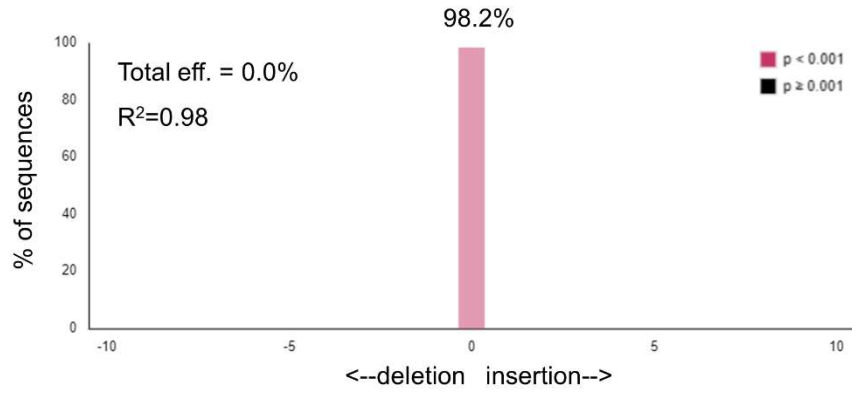
**F**

Treatment	Sample #	Editing efficiency (%)
CB2R-CRISPR	1	0.2
	2	0.6
	3	0.6
	4	0
	5	0
	6	0.2
Ctrl-CRISPR	1	1.4
	2	0.4
	3	0
	4	0
	5	0.1
	6	0.6

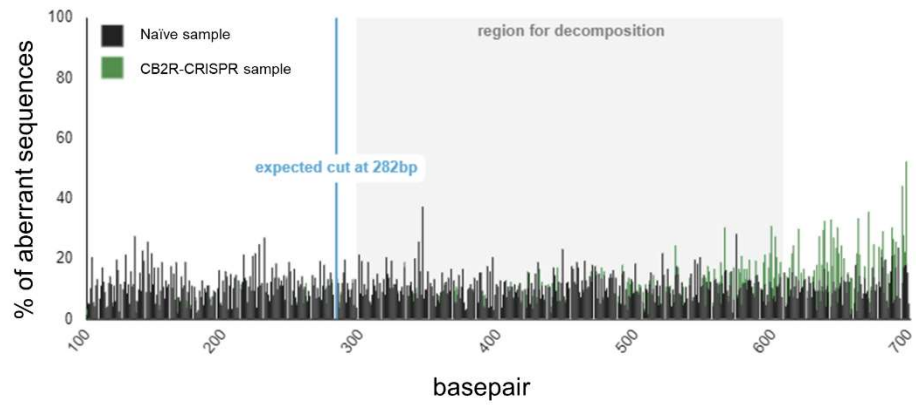
**Figure 25 Verifying the in vivo editing efficiency of *cnr2*-sgRNA/CRISPR system using TIDE analysis**

(A) Schematic diagram of TIDE analysis on sequence trace data. The sequence trace of test sample is decomposed by TIDE program into individual components, with the control sequence trace as a template to model the individual indel components. This figure is originally published on the TIDE analysis website (<http://tide.nki.nl>). (B and D) Representative indel spectrums and frequencies of CB2R-CRISPR- and Ctrl-CRISPR-edited VTA samples determined by TIDE analysis. (C and E) Representative examples of visualization of aberrant sequence signal in naïve (black) and test sample (green). The expected cut site (vertical dotted line) and the region used for decomposition (gray bar) were labelled on figures. (F) List of CRISPR editing efficiency in CB2R-CRISPR- and Control-CRISPR-edited samples.

**A** Indel Spectrum – LV-CB2R-CRISPR



**B** Aberrant sequence signal – LV-CB2R-CRISPR



**C**

Treatment	Sample #	Editing efficiency (%)
LV-CB2R-CRISPR	1	0.0
	2	0.0
	3	1.0

**Figure 26 Verifying the in vivo editing efficiency of lentivirus-mediated transfection of *cnr2*-sgRNA/CRISPR system using TIDE analysis**

(A) Representative indel spectrum and frequency of Lentivirus (LV)-mediated CB2R-CRISPR-edited VTA samples determined by TIDE analysis. (B) Representative example of visualization of aberrant sequence signal in naïve (black) and test sample (green). The expected cut site (vertical dotted line) and the region used for decomposition (gray bar) were labelled on figures. (C) List of CRISPR editing efficiency in LV-CB2R-CRISPR-edited samples.

## **CHAPTER 7: DISCUSSION**

### **7.1 Peripherally restricted CB1R agonist – a novel analgesic for cancer-induced bone pain**

Cancer-induced bone pain is one of the most common types of chronic pain in cancer patients, which presents in more than 30% of the cancer patients who have bone metastasis [34,173]. Currently, opioids are the primary medications for CIBP [35]. However, opioids are not always sufficient in pain management and are associated with severe adverse effects and contribute to the growing opioid epidemic and drug-related deaths [45,174–180]. Cannabinoids are considered a promising alternative analgesic to opioids, having demonstrated potent anti-allodynic effects in multiple chronic pain conditions, including CIBP [104–106,167,181–183]. Nevertheless, cannabinoids have had limited success in the clinic due to their central side effects including psychotropic effects induced by the activation of CB1Rs [157,167]. Recent studies demonstrate that activation of only peripheral cannabinoid receptors (both CB1Rs and CB2Rs) can produce significant antinociceptive effects on different chronic pain models [102,105,111,184–189]. Consistent with these studies, the use of peripherally restricted cannabinoids also exerts profound anti-allodynic effects on several neuropathic and inflammatory pain states [114–117]. Furthermore, limited central side effects were induced by these peripherally restricted agonists compared to typical cannabinoids [114–117].

In the present study, we evaluated the analgesic effect of a peripherally restricted selective CB1R agonist PrNMI in a murine model of CIBP. Our results show that an acute injection of PrNMI can effectively attenuate cancer-induced flinching but not guarding. This difference between the two pain behaviors may be due to the different pain states represented by the two pain behaviors. According to our observation, guarding is a behavior constantly presented in the cancer-bearing animals, suggesting the ongoing pain state. In contrast, flinching only occurs occasionally and sometimes is exacerbated by movement, which is closer to the expression of spontaneous pain. Associating the pain behavior results with these thoughts, it may suggest that acute PrNMI treatment is more effective in suppressing spontaneous pain than ongoing pain. However, further research is required to verify this idea. In addition to acute treatment, we also tested the analgesic effect of PrNMI after sustained administration (daily i.p. injection at a dose of 0.6 mg/kg). Excitingly, the repeated PrNMI treatment significantly alleviated both flinching and guarding in cancer-bearing animals. This enhanced analgesic effect induced by sustained PrNMI treatment may be the result of accumulating PrNMI in the animals. Indeed, PrNMI has a relatively long half-life (~7 hours) [116]. Importantly, our data has shown that PrNMI is less likely to induce tolerance compared to opioids as it can still exert great analgesic effect on cancer-bearing animals after 7 days of sustained administration compared to opioids, which gradually lose their efficacy after chronic treatment and may even induce hyperalgesia [190].

Although PrNMI was demonstrated as a peripherally restricted CB1R agonist in vivo, it was also shown to possess the ability to target CB2Rs in vitro. To determine whether the analgesic effect of PrNMI is mediated via CB1Rs or CB2Rs, we co-treated the animals with PrNMI and selective antagonists of CB1Rs/CB2Rs systemically. In consistent with the previous finding, PrNMI-mediated analgesia was significantly suppressed by blocking CB1Rs rather than CB2Rs [116], suggesting the analgesic effect of PrNMI is mediated by CB1Rs. Additionally, to confirm if PrNMI is peripherally restricted, we applied the antagonists of CB1Rs/CB2Rs spinally. In contrast to systemic treatment, spinal application of CB1R antagonist failed to block PrNMI analgesia demonstrating peripheral selectivity of the compound. Nevertheless, this conclusion may be weakened by the fact that the antagonists may not be able to distribute to the brain via spinal administration. Further studies that directly infuse the antagonists into the brain may be performed to finally confirm the peripheral selectivity of PrNMI.

## **7.2 Potential mechanisms underlying peripherally restricted CB1R agonist-mediated anti-nociceptive effect**

The exact mechanisms by which peripherally restricted CB1R agonists produce their anti-nociceptive effects on CIBP are still poorly understood but may involve the effects on primary afferents, bone remodeling and tumor growth. Cancer metastasis to the bone results in the damage and sprouting of primary afferent fibers, as well as inflammatory responses in the tumor-bone microenvironment, which activates nociceptive neurons and subsequently

produces pain [14,191]. CB1Rs are known to be expressed on the peripheral terminals of primary afferents [192], and are upregulated under multiple pathological conditions [112,113]. Previous studies showed that peripheral application of CB1R agonists or increase in endocannabinoids greatly attenuated both chronic cancer and non-cancer pain locally, as well as decreased spontaneous activity and sensitization of nociceptors [104,105,184–186,188,193], indicating the peripheral terminals of nociceptors are a critical site of cannabinoid-induced analgesia. By using a conditional peripheral CB1R knockout mouse strain, Agarwal, et al. nearly completely blocked the antinociceptive effect of systemically administered cannabinoids, thus demonstrating that cannabinoid-induced analgesia primarily occurs through the CB1 receptors distributed on peripheral terminals of nociceptors [111]. Our present study demonstrates that the activation of peripheral CB1Rs can effectively suppress CIBP in a mouse model of CIBP since the selective CB1R antagonist given spinally did not block the PrNMI analgesic effect while systemic administration significantly attenuated the effects. Based on these studies, it is likely that peripherally selective agonists activate CB1Rs on peripheral nociceptors, thus reducing CIBP. Further study would investigate if the knockout of CB1Rs on the nociceptors (like the previous study showed using a mouse strain that can specifically knock out CB1Rs in sensory neurons [111]) within the tumor-bone microenvironment may prevent the antinociceptive effect of peripherally restricted CB1R agonists.

Cannabinoid-induced bone remodeling may also contribute to its antinociceptive effects. Previous studies have shown that CB1Rs are expressed on

both osteoblasts, the bone-forming cells, and osteoclasts, the bone-resorbing cells although at low levels [34,163]. In contrast, the expression of CB2Rs is relatively higher particularly on osteoclasts [34,163]. The activation of CB2Rs was identified to produce anti-osteolytic effects in different animal models of bone loss. Studies by Lozano-Ondoua et al., demonstrated that CB2R agonism can result in a significant decrease in cancer-induced bone loss by directly inhibiting osteoclast activity, playing a role in the CB2 mechanism of antinociception [31]. Ofek et al. and Sophocleous et al. found that the activation of CB2Rs can inhibit age-related and ovariectomy-induced osteoporosis by promoting osteoblast differentiation and suppressing osteoclast function [194,195]. However, the functional role of CB1Rs in bone remodeling is still controversial. Previous studies by Tam et al. suggest the CB1Rs present on skeletal sympathetic nerve terminals promote bone formation by suppressing norepinephrine release [196]. Idris et al. reported that genetic deletion of CB1Rs prevents ovariectomy-induced bone loss by inhibiting the activity of osteoclasts [197]. Our chronic studies show that the peripherally restricted CB1R agonist PrNMI does not significantly reduce bone loss. This difference may be explained by the chronicity of receptor inactivation in CB1R-KO versus a 7-day agonist activity in our studies. The difference in mouse strains or the construct used for genetic mutation may also contribute to this inconsistency [198]. Lastly, as osteoblasts express CB1Rs as well [34,163], the inhibitory effect of PrNMI on these cells may counteract its antiosteolytic effect produced by suppressing osteoclasts.

The effect of PrNMI on cancer growth may also involve in the suppression of bone cancer pain. Tumor growth and proliferation directly promote bone degradation and pain exacerbation [31]. Numerous studies have demonstrated cannabinoids can affect cancer growth, although the reported effects are inconsistent. Studies have shown that the expression of cannabinoid receptors and the production of endocannabinoids were elevated in different types of cancer cells, which are frequently associated with the aggressiveness of tumor [199–201]. Additionally, in a murine skin cancer model, the absence of cannabinoid receptors resulted in a reduction in tumor genesis [202]. On the other hand, reduced expression of CB1Rs was shown to facilitate the growth of intestinal adenoma [203], and an increased endocannabinoid tone has been found to suppress the development of colon cancer [204]. In the present study, we found that the murine breast cancer cells we used to establish our bone cancer pain model express CB1Rs. Using an XTT cell proliferation assay, we found that the cell growth was not altered at all tested doses of PrNMI. These results suggest that PrNMI may not promote or suppress breast tumor growth by directly acting on cancer cells. However, whether PrNMI can modulate tumor growth still needs to be tested in vivo, as cannabinoids have been found to regulate angiogenesis and immune system [166], which are critical in tumor growth but cannot be reflected by in vitro assays.

### **7.3 Limited central side effects produced by peripherally restricted CB1R agonist PrNMI**

The psychotropic actions mediated by central CB1Rs represent the most troubling side effects that limit the clinical use of CB1R agonists [157,167]. Catalepsy, hypothermia, motor incoordination, and sedation are the classical indicators of central CB1Rs activation [157]. Here, we used open field test, rotarod test, rectal temperature test and ring immobility test to determine the CNS actions of PrNMI. Our data indicate that PrNMI does not induce motor incoordination in naïve mice. In addition, hypothermia is not seen at any of the antinociceptive doses but is detected at a 10-fold higher dose than the approximate analgesic ED<sub>50</sub> dose (0.1 mg/kg) (Figure 4 C). Catalepsy was not present at a dose of 0.3 mg/kg but was moderately-induced by PrNMI at 0.6 and 1.0 mg/kg as compared to our previous publication using a centrally acting CB1 agonist, WIN55,212-2 [187]. Interestingly, the open field test shows a decrease in total travel distance, but no differences was observed in tracking patterns, center time or moving time. While no published studies that have previously reported such findings, the idea that animals may have a reduction in overall travel distance but no significant change in activity on the rotarod, center time and most importantly moving time might suggest that the peripherally restricted cannabinoids may cause animals to move more in a stationary place. A review by Walsh and Cummins [205] described decreases in ambulation in an open field test typically indicates locomotor function and emotionality (anxiety/sedation); yet, our lack of an effect using rotarod and the no change in center time suggest no locomotor/sedation or anxiety activity,

respectively. Further studies using weight bearing test and elevated plus maze [206,207] may help confirm whether a peripherally restricted cannabinoid may result in an overall decrease in distance traveled and/or other changes in motor performance. Our previous pharmacokinetic findings in rats demonstrated minimal CNS access of PrNMI after systemic administration, particularly compared to other peripherally restricted CB1R agonists and prototypical cannabinoids [114–117]. However, it is essential to mention that all cannabinoid-associated central side effects were tested in naïve animals. Due to the potential increase of blood-brain barrier permeability in cancer-bearing animals [208], the expression profiling of the cannabinoid-associated central side effects may be altered. Further studies are required to determine the presentation of central side effects in cancer-bearing animals. Overall, current results suggest that PrNMI produces limited centrally mediated side effects.

#### **7.4 Other potential side effects associated with peripherally restricted CB1R agonist**

In addition to central adverse effects, other side effects produced by cannabinoids also limit the application of cannabinoids clinically. Hyperphagia is one of these commonly observed side effects. This side effect can induce an enhanced enjoyment and an intensification of the sensory and hedonic properties of food in cannabinoid users, leading to an over-consumption of highly pleasurable and energy-dense foods [168]. Currently, the mechanisms of this effect are not fully known but it is believed to be mediated via the activation of CB1Rs in the brain

reward circuitry [168]. In the present study, our data have shown that PrNMI did not increase the body weight, fat mass or lean mass in cancer-free mice, suggesting PrNMI may not induce hyperphagia. Interestingly, our results also exhibit that PrNMI suppressed the loss of body weight and increased the fat mass in cancer-bearing mice. These data implicate that PrNMI specifically induces hyperphagic actions in cancer-bearing mice. The mechanism underlying this phenomenon is unclear but may associate with the enhanced permeability of blood-brain barrier under cancer pain condition [208]. Although cannabinoid-induced hyperphagia is a problem for non-cancer cannabis users, this effect could be beneficial for cancer patients who commonly have reduced appetite and body weights in addition to bone pain [209].

Another side effect of cannabinoids could be constipation. Gastrointestinal (GI) tract has long been identified to express all elements in the endogenous cannabinoid system [210]. Cannabinoids affect GI motility mainly through CB1Rs expressed on enteric neurons [211]. The activation of these CB1Rs can inhibit the release of acetylcholine from enteric neurons, resulting in a decreased contractility and peristalsis of intestinal smooth muscle [212]. Our data is consistent with the inhibitory effects of CB1Rs in intestine as the administration of PrNMI significantly suppressed the ability to defecate in mice. This result indicates a concern for clinical application of peripherally restricted CB1R agonists. Interestingly, a self-reported survey shows that the prevalence of constipation was lower in recent marijuana users compared to those people who never use or use marijuana in the past [213]. This inconsistency between studies may be due to the complicated

ingredients of marijuana, which target other cannabinoid-associated receptors that can also regulate GI transit [210]. Further experiments are needed to confirm the actual reasons of this difference in GI activity.

### **7.5 Significance of using peripherally restricted CB1R agonist to treat cancer-induced bone pain**

The leading reason that CIBP remains a significant health problem today is the limited efficacy of analgesics available to treat this pain without impairing the patient's quality of life and the bone health of the patients. Opioid therapy is the primary treatment of moderate to severe bone cancer pain following cancer metastasis to bone [35]. Although opioids are very effective analgesics, they cause numerous unwanted side effects which limit the dose used. Recent studies by our group demonstrate that chronic morphine accelerates bone degradation in a murine model of sarcoma-induced bone loss [178]. Additionally, opioid analgesics cause a variety of psychotropic and life-threatening side effects, including somnolence, agitation, dizziness, cognitive impairment, hyperesthesia and respiratory depression [214]. As a result, the administration of opioids for CIBP significantly impairs the ability of patients to partake in daily events and effectively engage with their family and friends reducing their overall quality of life. Recently we have shown that the combination of analgesic therapies, CB2R agonist and a  $\mu$  opioid receptor agonist, for chronic pain can synergistically decrease the pain behaviors while also significantly reduce unwanted effects of both drugs [141]. In the present study, we identified a peripherally restricted CB1R agonist as a

promising alternative to the treatment of CIBP. Our results indicate that PrNMI can exert a profound analgesic effect on bone cancer pain and should be further tested in the presence of additional analgesics such as a CB2 agonist or an NSAID for cancer-induced pain. Importantly, PrNMI did not exacerbate cancer-induced bone destruction, did not enhance cancer proliferation and produced no severe side effects at therapeutic doses. Therefore, the use of peripherally restricted CB1R agonists in the treatment of CIBP is a highly favorable and safe alternative or at least a complement to current clinical therapy.

## **7.6 The influence of chronic morphine on the endogenous cannabinoid system in VTA**

In addition to the chronic pain epidemic, the opioid epidemic, as a result of chronic pain, is another severe public health issue that the United States is currently facing. According to the National Survey on Drug Use and Health published in 2017, over 11 million Americans were estimated to misuse prescription opioids and approximate 2 million of these people might have opioid use disorders [44]. Unfortunately, we currently have very limited ways to deal with this crisis, and the development of novel treatments are urgently needed.

Opioid-induced reward is the initial but critical step towards opioid addiction [50]. Inhibiting the development of opioid reward is considered as one of the strategies to deal with the opioid epidemic. Opioids induce reward by activating the brain reward circuitry. Ventral tegmental area (VTA) and nucleus accumbens (NAc) are two key brain loci in the reward circuitry that play a critical role in processing

reward-associated stimuli [49]. The dopaminergic neurons located in the VTA are thought to directly encode reward or a reward prediction signal by producing a rapid, phasic dopamine release in NAc [49]. However, the activity of these dopaminergic neurons is physiologically suppressed by tonic GABA input from VTA GABAergic neurons and other GABAergic neurons from the rostromedial tegmental nucleus (RMTg), NAc and ventral pallidum [49,50]. Opioids suppress this GABAergic tone by either direct hyperpolarization of the neurons or inhibition of neurotransmitter release, which eventually disinhibits the activity of dopaminergic neurons and allows for dopamine release [50]. This canonical two-neuron model is commonly thought, at least partially, as the neuronal mechanism controlling opioid-induced reward.

Although the two-neuron model provides a concise way to explain the process of opioid-induced reward, it has been demonstrated to be too simplified. In fact, opioids when used chronically can persistently alter the expression profiling of a variety of genes. Previous studies have shown that long-term opioid exposure changes the expression of multiple immediate early genes, including Arc, Fos, Egr1 and Egr2 in different brain regions related to reward processing [215,216]. The induction of these genes are able to rapidly alter synaptic activation and intracellular signaling with long-term changes in neurons [217]. Due to the development of the whole-genome sampling techniques in recent years, the ability to explore the alterations in gene expression profiling has been largely expanded [217]. With help of these techniques, many effector genes and gene families were found to be regulated by chronic opioid exposure, such as heat shock proteins,

inflammatory genes, opioid signaling, oxidative stress-related genes, etc [218–220].

Recently, the endogenous cannabinoid system has emerged as a hot topic in the study of opioid reward, since a large body of evidence has revealed this system participates in the initiation and development of opioid-induced reward [124,125,136,137,141,126–133]. However, compared to other molecular systems, relatively little is known about how chronic opioid exposure may affect endogenous cannabinoid system. To date, we have known that chronic opioid exposure significantly modulate the expression of CB1 receptors as well as the production of endocannabinoids, AEA and 2-AG, in various reward-associated brain regions, including nucleus accumbens [118–123,134,135]. To expand our understanding about how chronic opioid modulates endogenous cannabinoid system, we sought to investigate the alterations of cannabinoid receptors and endocannabinoids in VTA after chronic opioid treatment.

To investigate the effect of chronic opioid, we gave the animals sustained treatment of morphine at a dose of 5 mg/kg (i.p.) for 5 consecutive days [151]. Our study reveals that chronic morphine treatment does not change the production of either AEA or 2-AG in the VTA nor the expression and activity of the enzymes DAGL $\alpha$  and MAGL responsible for the synthesis and degradation of 2-AG, respectively. However, we found that acute morphine significantly reduced AEA production in both chronic morphine- and saline-treated rats, suggesting that opioids may induce an acute and reversable regulation of AEA production in the VTA. The actual significance for this phenomenon is unclear and no other study

has reported this result. Considering the relative higher affinity of AEA to CB1Rs than to CB2Rs as well as the presynaptic localization of CB1Rs, this reduction of AEA after morphine treatment may reflect a negative feedback mechanism in opioid reward. Furthermore, we also found that chronic morphine can reduce the expression of CB2Rs but not CB1Rs in VTA. Overall, our studies suggest that chronic morphine significantly reduces the expression of CB2Rs in the VTA but does not alter the production of 2-AG or AEA, the expression of DAGL $\alpha$ , MAGL and CB1Rs.

Long-term opioid exposure mediates persistent alterations in the expression of a variety of genes associated with the function of reward-processing networks in the brain, resulting in opioid reward and addiction [217]. These alterati

However, many other potential cannabinoid receptors, endocannabinoids and related enzymes in the endogenous cannabinoid system are not well explored, which could be regulated by opioids. Thus, further research, such as proteomic analysis, is needed to have a comprehensive understanding of the whole endogenous cannabinoid system in VTA.

### **7.7 Modulatory effects of 2-AG and CB2Rs on chronic morphine-induced reward**

As we mentioned previously, the endogenous cannabinoid system has emerged as a hot topic in the study of opioid reward. Indeed, CB1Rs were found to be colocalized with MORs in multiple brain regions and even form heterodimeric system with each other [221,222]. Furthermore, chronic administration of

cannabinoids, such as  $\Delta$ 9-THC, significantly increased the gene expression of endogenous opioids, including proenkephalin and prodynorphin [223–226], and regulated MOR expression and corresponding downstream signaling [222,227].

Importantly, endogenous cannabinoid system was demonstrated to modulate opioid-induced reward. Previous studies have shown that blockade of CB1R using selective antagonists attenuates opioid-induced conditioned place preference and self-administration [124–126,128–133]. AEA has also been investigated intensively, although the modulation of this endocannabinoid does not seem to alter the reinforcing effect of heroin or the effects of morphine on VTA dopamine neurons [136,137]. Compared to CB1Rs and AEA, 2-AG is barely investigated in opioid-induced reward. To date, no study has reported the role of 2-AG in opioid reward. Our results found that enhancing 2-AG tone by a selective MAGL inhibitor MJN110 significantly attenuated chronic morphine-induced preference. Interestingly, we also saw that the saline-treated rats presented a preference to the non-drug-paired chamber after MJN110 treatment although no significant difference was observed compared to the rats in the vehicle-saline group. These studies indicate that the inhibitory effect of MJN110 on chronic morphine-induced reward may result from its aversive effect. Indeed, the endogenous cannabinoid system has been implemented in the aversion-associated brain regions and can regulate aversive behaviors [228,229]. However, the results still did not exclude the possibility that 2-AG may also regulate reward process by targeting VTA, since previous studies have shown that 2-AG can enhance dopamine release in NAc Shell [142,143]. In future studies, the

manipulation of 2-AG production in reward-associated brain regions, such as VTA, is needed in order to know if 2-AG may participate in reward processes.

Like 2-AG, very little is known about the modulatory role of CB2R in opioid reward. Currently, only one study has investigated this topic and showed that the activation of CB2Rs inhibits acute morphine-induced reward [141]. Our studies have revealed that the activation of CB2Rs with a selective CB2R agonist JWH015 significantly decreased chronic morphine-induced reward. Different from 2-AG, CB2R activation did not change the conditioned place preference in saline-treated rats, implicating that CB2Rs exert inhibitory effect on reward processing. To further explore whether this inhibitory effect is via the CB2Rs located in VTA, we directly knocked down the VTA CB2Rs using the genomic editing technique CRISPR/Cas9. However, no significant difference in morphine-induced preference was observed between the CB2R-knockdown group and the control group. This result indicates that the inhibitory effect of CB2Rs on chronic opioid-induced reward may not be mediated via the CB2Rs in VTA. Additionally, it is also possible that the CRISPR/Cas9 system did not work effectively *in vivo*. To exclude this possibility, we examined the editing efficiency of CRISPR/Cas9 using TIDE analysis. Unexpectedly, the results showed that the editing efficiency in all rats is extremely low and does not seem to be different from control group. One possible reason for the low editing efficiency is the low transfection efficiency of plasmid injection. Therefore, transfection using the CRISPR system in the VTA with lentivirus was performed. Unfortunately, the editing efficiency was also very low. In future steps, the direct injection of CB2R antagonists into VTA, instead of using CRISPR

technique, will be performed to explore whether VTA CB2Rs may modulate chronic morphine-induced reward.

## **7.8 Potential mechanisms of endocannabinoid-mediated regulation of opioid reward and future directions**

Although the studies are still ongoing, current evidence supports the idea that the endocannabinoid system participates in opioid-induced reward. The next important research question would be what the molecular and cellular mechanisms underlying this process are.

CB1Rs, due to their presynaptic localization, are believed to serve as an autoreceptor in the central nervous system [230]. Together with the postsynaptic production of endocannabinoid 2-AG and the presynaptic distribution of 2-AG degrading enzyme MAGL, CB1Rs and 2-AG are considered to form a regulatory pathway and modulate neuronal transmission in a retrograde manner [51,80]. This special regulation manner may also be adapted in the endocannabinoid system-mediated modulation of opioid reward. Indeed, previous studies have found CB1Rs are abundantly expressed on synaptic terminals targeting dopaminergic neurons in the VTA [231]. Rashidy-Pour et al. have demonstrated that specific blockade of CB1R in VTA significantly inhibited morphine-induced rewarding behavior, which supported the idea that CB1R working as an autoreceptor disinhibits GABAergic suppression of dopaminergic neuron activity [131]. Furthermore, the 2-AG biosynthetic enzyme DAGL is identified in dopaminergic neurons, whereas the degrading enzyme MAGL is found in presynaptic terminal

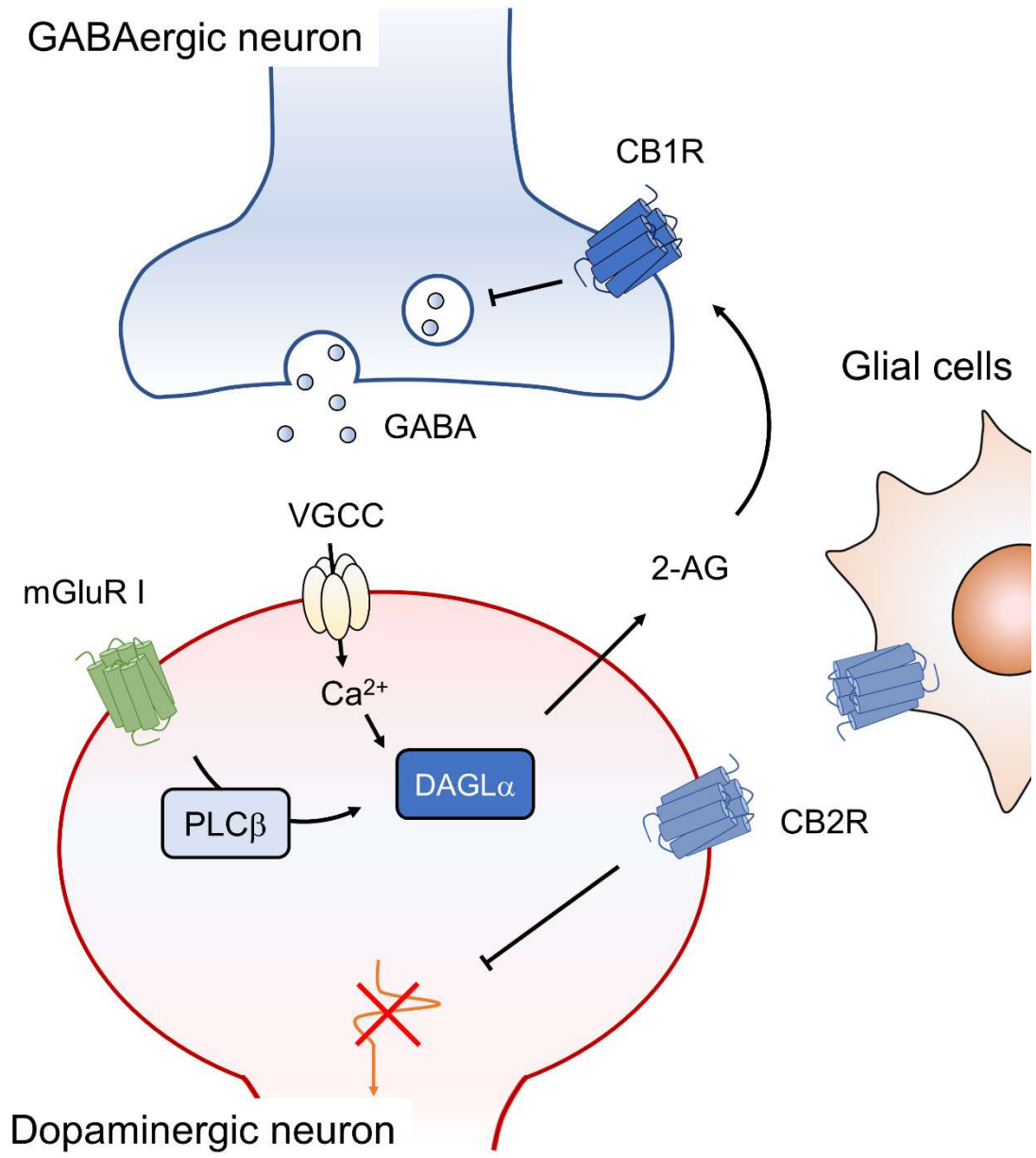
[232]. All these studies have strongly suggested the involvement of the 2-AG/CB1R-mediated retrograde regulation in opioid reward. Interestingly, our microdialysis data revealed that both acute and chronic morphine treatments did not change the production of 2-AG in the VTA. The actual reasons for this inconsistency are unknown, but one of the reasons could be the utility of alternative non-dopaminergic reward pathways in animals. As Nader and van der Kooy have shown, the opioid reward in opioid-naïve and opioid-dependent but non-withdrawal rats is mediated via a non-dopaminergic reward pathway through tegmental pedunculo-pontine nucleus [233]. The animals we tested in the present study may fall into a similar state, explaining why no alteration was observed. In the future study, investigating the modulatory effect of opioid on endocannabinoid production may need to consider the potential influence of the animals' pathological state(s).

The studies of CB2R in opioid reward have always been unappreciated due to the low expression of this receptor in the central nervous system until recently this receptor was demonstrated to be functionally expressed in the central nervous system [59,234]. In contrast to CB1Rs, CB2Rs are primarily located on postsynaptic neurons, which suggests an inhibitory role of this receptor in reward process. This hypothesis seems to be true in cocaine-induced reward since the administration of a selective CB2R agonist JWH133 significantly attenuated the activity of dopaminergic neurons and dopamine release in the NAc shell in the presence of cocaine [59,234]. Currently, this is being validated in opioid-induced reward [141]. ABHD6 and ABHD12 are two new enzymes that were recently found to hydrolyze 2-AG [87]. However, as they are only responsible for ~20% of the 2-

AG hydrolysis in the brain [87], the importance of these two enzymes in drug reward is poorly characterized. A recent study found that ABHD6 is postsynaptically expressed in adult mouse cortex and its selective inhibition induced CB1R-dependent long-term depression by subthreshold stimulation [235]. ABHD12, in addition to hydrolyzing 2-AG, was identified to regulate the accumulation of proinflammatory lipids that promote microglial and neurobehavioral abnormalities [236]. These studies have indicated the important roles of ABHD6 and ABHD12 in neuromodulation, which may also involve in the regulation of opioid reward.

To date, opioid-induced neuronal adaptations remain the major focus of current studies in opioid reward, but accumulating evidence reveals that the non-neuronal glial cells are also responsible for the rewarding effect produced by opioids [237]. Glial cells, especially microglia, are the immune cells and key regulators in the central nervous system. As numerous studies have shown that they participate in the regulation of a variety of CNS events [238,239], it is not surprising to know that they are also active players in opioid reward. In fact, opioids can directly stimulate microglial cells and lead to their functional conversion, which eventually results in the modulation of synaptic transmission and plasticity that are involved in opioid reward [237]. These studies suggest that interrupting the opioid-induced glial activation could be potential therapeutics in the treatment of opioid reward. CB2Rs are expressed by microglia in the brain, particularly under pathological conditions [240]. As this type of receptor commonly couples with inhibitory intracellular signaling and exert immunosuppressive effect in the

periphery [52], one can speculate that the activation of CB2Rs on glial cells will inhibit the opioid-induced glial activation and subsequently rewarding effect. Currently, several studies did show that CB2R activation suppresses opioid-induced glial activation [151,241]. However, the microglia cells observed in these studies are either cultured in vitro or not derived from brain, which may not directly reflect the exact responses of microglial cells in the VTA. Also, whether this CB2R-mediated suppression of glial activation may relieve opioid reward is still needed to be tested in animals especially those with no expression of CB2R in microglia.



## **Figure 27 Potential mechanisms of endocannabinoid-mediated regulation of opioid reward**

Following postsynaptic activity, the altered intracellular signaling, such as enhanced Ca<sup>2+</sup> influx and the activation of Gq/11-coupled GPCRs, promotes the production of endocannabinoids, particularly 2-AG, from postsynaptic neurons. These endocannabinoids then travel retrogradely and activate presynaptic CB1Rs to suppress the release of neurotransmitters at presynaptic terminals. CB2Rs expressed on neurons may suppress the activation of dopaminergic neuron firing. Additionally, CB2Rs expressed on glial cells may inhibit glial activation-mediated regulation of opioid reward. mGluR I, metabotropic glutamate receptor type I; VGCC, voltage-gated calcium channel.

## **7.9 Conclusions and significance**

In the present study, we demonstrated that targeting the peripheral CB1Rs with peripherally restricted CB1R agonist can effectively attenuate cancer-induced bone pain in a syngeneic murine model and results in limited central side effects, as well as other cannabinoid-associated side effects. These results suggest that targeting peripheral CB1Rs can be a valuable therapeutic strategy for treating cancer-induced bone pain. To date, the peripherally restricted CB1R agonists have not been tested clinically, but a recent clinical trial evaluating the therapeutic effects of Dronabinol (a specific form of THC) on bone cancer pain and reducing opioid use in patients with metastatic breast cancer has shown promising results (NCT03661892, data not shown), providing the possibility that peripherally

restricted CB1R agonists can be effective treatments for bone cancer pain while reducing central adverse effects.

Our current study also found that the elevation of 2-AG tone and activation of CB2Rs remarkably inhibits chronic morphine-induced reward behavior. Yet, chronic morphine exposure reduces the expression of CB2Rs but does not significantly change the production of 2-AG or the expression of the enzymes, DAGL $\alpha$  and MAGL, responsible for the synthesis and degradation of 2-AG in the VTA. Overall, these data provide the possibility that targeting endogenous cannabinoid system, particularly 2-AG and CB2R signaling, may create therapeutic opportunity for opioid addiction.

## APPENDIX A: LIST OF PUBLICATIONS

### Peer-reviewed Publications

1. **Zhang, H.**, Moutal, A., Smith, A.F., Khanna, R., Largent-Milnes, T.M., and Vanderah, T.W. The effects of chronic morphine-induced alteration in VTA endogenous cannabinoid system on opioid reward. In preparation.
2. **Zhang, H.**, Largent-Milnes, T.M., and Vanderah, T.W. Glial neuroimmune signaling in opioid reward. *Brain Research Bulletin*, 2019;155:102-11.
3. **Zhang, H.**, Lund, D.M., Ciccone, H.A., Staatz, W.D., Ibrahim, M., Largent-Milnes, T.M., Seltzman, H.H., Spigelman, I. and Vanderah, T.W. A peripherally restricted cannabinoid 1 receptor agonist as a novel analgesic in cancer-induced bone pain. *Pain*, 2018;159(9):1814-23.
4. Grenald, S.A., Doyle, T.M., **Zhang, H.**, Slosky, L.M., Chen, Z., Largent-Milnes, T.M., Spiegel, S., Vanderah, T.W. and Salvemini, D. Targeting the S1P/S1PR1 axis mitigates cancer-induced bone pain and neuroinflammation. *Pain*, 2017;158(9):1733-42.
5. Forte, B.L., Slosky, L.M., **Zhang, H.**, Arnold, M.R., Staatz, W.D., Hay, M., Largent-Milnes, T.M. and Vanderah, T.W. Angiotensin-(1-7)/Mas receptor as an antinociceptive agent in cancer-induced bone pain. *Pain*, 2016;157(12):2709-21.

### Abstracts

1. **Zhang, H.**, Lund, D.M., Coleman, D., Davidson-Knapp, R.B., Ciccone, H.A., Staatz, W.D., Ibrahim, M.M., Largent-Milnes, T.M., Seltzman, H.H., Spigelman, I.,

and Vanderah, T.W. Targeting peripheral CB1 receptors and central CB2 receptors in chronic pain and addiction. Society for Neuroscience, 2018, San Diego, CA

2. **Zhang, H.**, Lund, D.M., Ciccone, H.A., Staatz, W.D., Ibrahim, M.M., Largent-Milnes, T.M., Seltzman, H.H., Spigelman, I., and Vanderah, T.W. Treating cancer-induced bone pain with a peripherally restricted agonist for cannabinoid receptor 1. AZBio Awards, 2018, Phoenix, AZ

3. **Zhang, H.**, Lund, D.M., Ciccone, H.A., Staatz, W.D., Largent-Milnes, T.M., Spigelman, I., and Vanderah, T.W. Treating cancer-induced bone pain with a peripherally selective agonist for cannabinoid receptor 1. Junior Investigator Poster Forum, 2017, Tucson, AZ

4. Cottier, K.E., Galloway, E.A., **Zhang, H.**, Tome, M., Schaefer, C., Calabrese, E., Kim, J., Vanderah, T.W., Davis, T.P., and Largent-Milnes, T.M. Blood-brain barrier integrity is temporally dysregulated in a spreading depression induced model of episodic headache. Junior Investigator Poster Forum, 2017, Tucson, AZ

5. Lund, D.M., **Zhang, H.**, Largent-Milnes, T.M., and Vanderah, T.W. Using a peripherally-restricted cannabinoid to treat cancer-induced bone pain. Undergraduate Research Opportunity Consortium, 2017, Tucson, AZ

6. **Zhang, H.**, Doyle, T.M., Largent-Milnes, T.M., Jacobson, K.A., Salvemini, D. and Vanderah, T.W. Targeting A3 adenosine receptor in HIV-1 gp120-induced neuropathic pain. 36th Annual Scientific Meeting of American Pain Society, 2017, Pittsburgh, PA

# APPENDIX B: HUMAN/ANIMAL SUBJECTS APPROVAL



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## Verification of Institutional Animal Care and Use Committee (IACUC) Review and Approval

This protocol has been reviewed by the IACUC and the work may commence at this time. This approval only authorizes the activities reviewed by the IACUC as described on the final version of the protocol.

Principal Investigator: **Vanderah, Todd W**  
Department: **Pharmacology**

Protocol Number: **06-110**  
Title: **Identification of analgesic compounds to treat acute and chronic pain**

Approval Date: **07/19/2018**  
Expiration Date: **07/19/2021**

Funding Sources: **Departmental  
NIH/NCI R01 CA142115  
NIH/NIDA P01 DA041317; R01 DA043543  
Proneurogen  
Regulonix**

Grant to Protocol Review: **Congruent**

Additional Notes Concerning Submission: **None**

**The Principal Investigator (PI) is responsible for all work conducted on this protocol. As such, the PI must ensure that:**

- The protocol will be conducted in accordance with all applicable federal and institutional policies, procedures and regulations, including the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), the Guide for the Care and Use of Laboratory Animals, and all relevant institutional regulations and policies regarding animal care and use at the University of Arizona.
- All procedures involving animals will be carried out humanely and as described in the approved protocol.
- IACUC approval will be secured before initiating any change in the study design or procedures listed on this protocol. Protocol participants understand that all amendments must be approved by the IACUC prior to implementation.
- Work performed without IACUC approval is not published with certification of IACUC approval.
- All individuals working autonomously on this protocol are qualified to conduct procedures involving animals, are competent in the techniques cited in the protocol, and will maintain



appropriate and complete animal records. All untrained staff will be sufficiently supervised until competency is achieved.

- The IACUC is notified regarding any unanticipated deaths or unexpected study results or a phenotype that negatively impacts the welfare of the animals, including but not limited to those that require veterinary care or treatment not described in the approved protocol.
- The hazards listed on this protocol will be handled as per Research Laboratory Safety Services (RLSS) guidelines and stipulations. Work with hazards is not performed until RLSS approval has been granted.



Sean W. Limesand, PhD  
IACUC Chair



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